Visualization of microbes involved in soil methane dynamics

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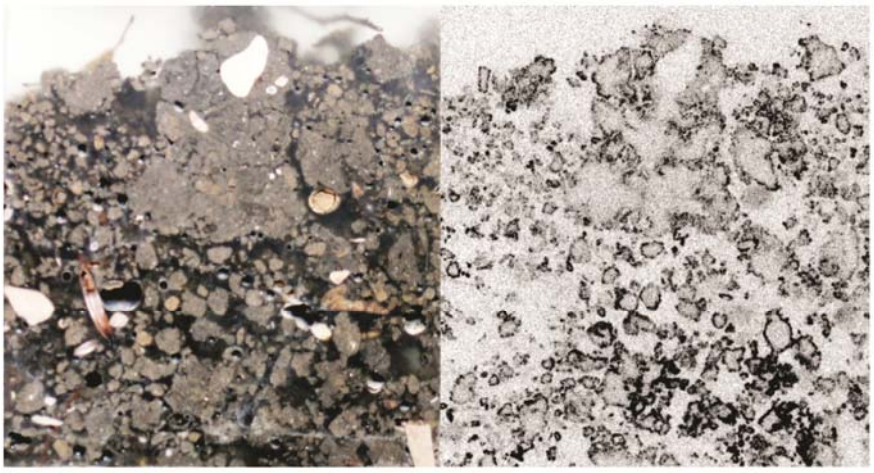
Dissertation for the degree of doctor of sciences



ETH Zurich, 2010

DISS. ETH N^{o}. 19128

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A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

presented by

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2010

Für alle, die an dieser Arbeit interessiert sind
und alle, die von dieser Arbeit profitieren können.

Für meine Familie,
und alle, diese Arbeit und mich unterstützt haben.

Ganz besonders für meinen Grossvater Edwin J. Heizmann (1894-1996)
und alle, die an mich geglaubt haben.

To those who are interested in this work
and to those who might profit from this work.

To my family
and to those who supported me and this work.

Especially to my grandfather Edwin J. Heizmann (1894-1996)
and to those who believed in my abilities.

Contents

Abstract

Aerobic soils can contain microbes capable of assimilating methane (CH_4). Under anaerobic conditions, other soil microbes might produce CH_4. If soil CH_4 uptake exceeds CH_4 emissions, the soil is defined as a CH_4 sink. Worldwide, soils acting as CH_4 sinks are accounted to assimilate up to 10% of natural and anthropogenic CH_4 emissions from the atmosphere. The terrestrial CH_4 sink is important to be maintained in terms of climate regulation because CH_4 is one of the most important greenhouse gases. Uptake of atmospheric CH_4 depends on soil moisture and soil texture. Nitrogen fertilization and soil acidification might significantly reduce CH_4 assimilation by the microbes involved (methanotrophic bacteria). However, the mechanisms regulating CH_4 uptake are only partially understood. Interactions between soil methanotrophs and their environment are complex as soil physical parameters and chemical components vary. Consequently, estimations of the terrestrial CH_4 sink under future conditions and recommendations for managing soils in order to maintain the soil CH_4 sink are complicated.

In this work, we studied assimilation of atmospheric CH_4 in the soil. The aim was to study spatial distributions of CH_4 assimilation in different ecosystems and how these distributions change under treatments such as drought, liming and nitrogen fertilization. By additionally measuring net CH_4 flux rates, the CH_4 uptake regulating mechanisms underlying ecosystem level effects of treatments were studied.

The development of a new approach allowed for the first time to study the spatial distribution of CH_4 assimilation in the intact soil structure. In brief, microbes in undisturbed soil cores were labelled with $^{14}CH_4$. Samples were subsequently freeze-dried and embedded in epoxy resin. Casted samples were sawn in vertical oriented soil sections. Finally, autoradiographic images were generated having a resolution of 50 µm. This method was applied to several forest soils (*Alptal* and *Lägern*) and grassland soils (*Furka*, *Alp Weissenstein* and *Früebüel*) in Switzerland to investigate the impacts of soil structure, pH and bedrock on CH_4 uptake. Moreover, the effect of NH_4^+ based fertilizers was investigated by comparing results from fertilized and unfertilized plots in three experiments: 1. Ammonium nitrate (NH_4NO_3) was added to

locally collected rain water which then was sprinkled over the ground vegetation of a forest soil at *Alptal* during more than ten years. 2. Urine from cattle and NH_4NO_3 were applied during two years to two grasslands (*Alp Weissenstein* and *Früebüel*), while in half of the plots summer drought was simulated by the installation of rain shelters. The effect of the amount of these fertilizers has been tested with a gradient experiment at *Früebüel*. 3. Ammonium sulphate ($(NH_4)_2SO_4$), sodium nitrate ($NaNO_3$) and manure were applied for more than hundred years to grassland plots (Park Grass experiment at *Rothamsted,* UK) which contain a pH gradient (pH 4.0-7.0) as a consequence of liming. This pH gradient was also achieved in unfertilized soil at the Park Grass experiment. Net CH_4 flux rates have been measured in the laboratory and additionally *in situ* at *Alptal, Furka* and at the Park Grass experiment.

The spatial distribution of CH_4 uptake in unfertilized soils depended mainly on soil aggregation and soil moisture. Drought significantly increased CH_4 uptake at *Früebüel* and *Alp Weissenstein* and the CH_4 assimilation at the bottom of the soil cores was increased in the dry compared to the moist samples from *Früebüel*. Grassland soils showed a distinct maximum in CH_4 uptake at 2.5–5.0 cm depth, while maximum CH_4 assimilation in forest soils occurred in 4.0-10.0 cm depth. Increased amounts of CH_4 assimilated at the edges of aggregates (especially in forest soils), along pores, in some porous stones and around limestone rocks. Roots did not directly affect CH_4 uptake. Hardly any CH_4 assimilated in humus layers. Results from the fertilizer experiments demonstrated that ammonium rather than nitrate reduced CH_4 uptake. However, effects only occurred when soils were either acidified or dry, probably due to low nitrification rates and little NH_4^+ uptake by plants under these conditions, respectively. Liming was found to prevent negative effects of NH_4^+ based fertilizers on CH_4 uptake in moist soils. Inhibition of CH_4 uptake by fertilizers was frequently restricted to only a part of the soil which might explain controversial findings of previous studies which did not consider the spatial dimension of fertilizer effects. Under dry conditions at *Alp Weissenstein* and *Früebüel*, nitrogen fertilizers inhibited CH_4 uptake in the subsoil, while below, simultaneously CH_4 uptake increased. *In situ* measured CH_4 flux results from a partner project suggest that this shift of the CH_4 assimilating soil layer did not affect the CH_4 sink of the entire soil.

Our results suggest that summer droughts which are predicted to occur more frequently in Switzerland in the next decades will rather increase than decrease CH_4 uptake in Swiss grassland soils. However, nitrogen fertilizers should not be applied to very dry soils in consecutive years, as continuous treatments might increase the fraction of the soil in which CH_4 uptake is inhibited, thus reducing net CH_4 uptake of the soil. High amounts of ammonium releasing fertilizers should rather be applied in organic forms than in inorganic forms to minimize interference with the soil CH_4 sink.

Zusammenfassung

Aerobe Böden können Mikroorganismen enthalten, welche atmosphärisches Methan (CH_4) assimilieren. Unter anaeroben Bedingungen können andere Bodenmikroben CH_4 produzieren. Je nachdem ob die CH_4 Aufnahme oder die CH_4 Emissionen eines Bodens überwiegen, ist der Boden als CH_4 Senke oder als CH_4 Quelle definiert. Böden, die als CH_4 Senken wirken, nehmen bis zu 10% des weltweit durch natürliche und anthropogene Prozesse emittierten CH_4 aus der Atmosphäre auf. Die Erhaltung der Bodensenke für CH_4 ist bedeutend für die Klimaregulierung, weil CH_4 eines der wichtigsten Treibhausgase ist. Wassergehalt und Bodenstruktur regulieren die terrestrische CH_4 Aufnahme aus der Atmosphäre. Stickstoffdüngung und Bodenversauerung können die CH_4 Assimilation durch Mikroorgansimen (methanotrophe Bakterien) erheblich senken. Die regulierenden Mechanismen der atmosphärischen CH_4 Aufnahme in Böden sind jedoch nur teilweise bekannt. Die Interaktionen zwischen methanotrophen Bakterien und ihrer Umwelt sind komplex, da insbesondere physikalische Parameter und chemische Stoffe im Boden variieren. Aufgrund dessen ist es besonders schwierig abzuschätzen, wie terrestrische CH_4 Senken auf ändernde Umweltbedingungen reagieren und Empfehlungen für eine nachhaltige Bodenbewirtschaftung in Bezug auf die CH_4 Senke zu gestalten.

In dieser Arbeit wurde die Aufnahme von atmosphärischem CH_4 in Böden untersucht. Das Ziel dieser Dissertation bestand darin, die räumliche Verteilung der atmosphärischen CH_4 Assimilation in Böden verschiedener Ökosysteme zu erforschen und zu untersuchen, wie sich diese Verteilungen unter Trockenheit, Boden-Kalkung und Stickstoffdüngung ändern. Die hier beschriebenen Experimente wurden durchgeführt, um das Verständnis der Regulierung der CH_4 Senkenfunktion von Böden zu verbessern. Deswegen wurden zusätzlich CH_4 netto Flussraten der Böden gemessen.

Mit der Entwicklung eines neuen Ansatzes gelang es erstmals, die Verteilung der CH_4 Aufnahme im intakten Bodengefüge zu erfassen und darzustellen. Dabei wurden die methanotrophen Bakterien in ungestörten Bodenproben mit $^{14}CH_4$ markiert. Anschliessend wurden die Proben gefriergetrocknet in Epoxydharz eingebettet und in

vertikale Scheiben gesägt. Von diesen wurden schlussendlich Autoradiographien mit einer Auflösung von 50 µm hergestellt. Mit diesem Ansatz wurde die räumliche Verteilung der CH_4 Aufnahme in Proben verschiedener Schweizer Wald- (*Alptal* und *Lägern*) und Graslandböden (*Furka, Alp Weissentein und Früebüel*) mit Berücksichtigung der Bodenstruktur, des pH und des Gesteins untersucht. Ausserdem wurde der Einfluss von Stickstoffdüngern auf die CH_4 Aufnahme in drei Experimenten untersucht: 1. In einem Wald in *Alptal* wurde während mehr als zehn Jahren dem lokal gesammelten Regenwasser Ammoniumnitrat (NH_4NO_3) beigefügt und dieses mit Sprinklern ausgebracht. 2. Auf zwei Wiesen (*Früebüel* und *Alp Weissenstein*) wurden während zwei Jahren Urin von Kühen, sowie NH_4NO_3 ausgebracht. Zusätzlich wurde auf der Hälfte der Plots Trockenheit durch Regendächer simuliert. Darüber hinaus wurde auf *Früebüel* die Wirkung der Düngermengen in einem Gradientenexperiment untersucht. 3. Im Park Grass experiment in *Rothamsted*, England wurden während mehr als 100 Jahren die Dünger Ammoniumsulfat ($(NH_4)_2SO_4$), Natriumnitrat ($NaNO_3$) und Mist ausgebracht. Die Düngerfelder und der Kontrollstreifen enthalten zudem einen durch Kalkung eingestellten pH Gradienten (pH 4,0-7,0). Von allen Proben wurden die netto CH_4 Flussraten im Labor gemessen und auf den Standorten *Alptal*, *Furka*, und im Park Grass Experiment zudem *in situ* bestimmt.

Die Resultate zeigten, dass in ungedüngten Böden überwiegend Bodenstruktur und Wassergehalt die räumliche Verteilung der CH_4 Aufnahme beeinflussten. Trockenheit erhöhte die CH_4 Aufnahme auf *Früebüel* und *Alp Weissenstein* und führte in Proben von *Früebüel* zu einer Tiefenerweiterung der CH_4 aufnehmenden Bodenschicht. Wiesenböden nahmen am meisten CH_4 in 2,5-5,0 cm, Waldböden in 4,0-10,0 cm Tiefe auf. Erhöhte Mengen an CH_4 wurden an Aggregaträndern (insbesondere in Waldböden), entlang Bodenporen, in porösen Steinen sowie um Kalksteine assimiliert. Wurzeln hatten keinen direkten Einfluss auf die CH_4 Aufnahme. In Humusschichten wurde kaum CH_4 assimiliert. Aus den Düngerexperimenten ging hervor, dass Ammonium die CH_4 Aufnahme stärker inhibiert als Nitrat. Die inhibierenden Effekte der Dünger traten jedoch nur im Zusammenhang mit Bodenversauerung oder bei Trockenheit auf, wahrscheinlich weil die Nitrifikationsraten mit sinkendem pH abnehmen und Pflanzen bei Trockenheit weniger NH_4^+ aufnehmen. Das Kalken verhinderte die Reduktion der CH_4

Assimilation durch Ammonium in feuchten Böden. Die Inhibierung der CH_4 Aufnahme betraf häufig nur einen Teil des Bodens, was möglicherweise kontroverse Resultate bisheriger Effektstudien erklärt, die die räumliche Dimension der Effekte nicht berücksichtigt hatten. Unter Trockenheit inhibierte die Stickstoffdüngung auf *Alp Weissenstein* und *Früebüel* die CH_4 Aufnahme im Oberboden, worauf sich die CH_4 Aufnahme in der darunterliegenden Bodenschicht erhöhte. Diese Verschiebung der CH_4 assimilierenden Schicht im Boden hatte gemäss *in situ* Messungen, die in einem Partnerprojekt durchgeführt wurden, keine Konsequenzen für die CH_4 Senke des Bodens.

Aufgrund der vorliegenden Resultate dieser Studie erwarten wir, dass unter dem Einfluss von Sommerdürren, die bei fortschreitendem Klimawandel in der Schweiz wahrscheinlich häufiger auftreten werden, die CH_4 Aufnahme von Schweizer Wiesenböden eher erhöht wird. Allerdings sollte es vermieden werden Stickstoffdünger wiederholt auf sehr trockene Böden auszubringen, da dadurch möglicherweise eine Inhibierung der Methanaufnahme in weiteren Teilen des Bodens auftritt. Grosse Mengen an Düngern, die Ammonium freisetzen, sollten eher in organischer als in anorganischer Form ausgebracht werden, um die CH_4 Aufnahme nicht zu beeinflussen.

Chapter 1

Introduction

1.1 General introduction

Methane and global warming

Methane (CH_4), one of the major greenhouse gases in the atmosphere besides carbon dioxide (CO_2) and water vapour, contributes largely (20%) to current increases in global surface temperatures (Forster *et al.* 2007). The global warming potential of CH_4 is up to 33 times higher compared to CO_2 when calculated over a time horizon of 100 years (Boucher *et al.*, 2009; Shindell *et al.*, 2009). According to measurements from ice cores, CH_4 concentrations in the atmosphere were almost constant for more than 400'000 years before 1760 (Petit *et al.*, 1999). However, over the past 250 years, atmospheric CH_4 concentrations have almost doubled and are currently estimated to be 1774 parts per billion (ppb) (Forster *et al.* 2007). Therefore, improved knowledge about CH_4 sinks and sources is urgently needed to assess CH_4 mitigation potentials.

Sources and sinks of CH_4

CH_4 is released to the atmosphere from both, natural and anthropogenic sources at approximately 600 Tg yr^{-1} (Lelieveld *et al.*, 1992). Major sources of CH_4 are natural wetlands, tundra soils and rice paddies (38%), ruminants (19%) and the energy sector including coal mining, natural gas production and transportation (18%). Landfills, burning of biomass and termites are further, but minor sources of CH_4 (<10%, each; Denman *et al.,* 2007).

Simultaneously, three main CH_4 sinks exist, which all together remove up to 580 Tg CH_4 yr^{-1} from the atmosphere (Lelieveld *et al.*, 1992). These CH_4 sinks are: (1) tropospheric hydroxyl (OH) radicals, (2) stratospheric loss and (3) soils, which are estimated by the *Intergovernmental Panel on Climate Change* (IPCC) to contribute 88%, 7% and 5% to the total CH_4 sink, respectively (IPCC, 2001; Denman *et al.*, 2007). However, estimates of the individual CH_4 sinks regarding the total CH_4 uptake differ in numerous studies, e.g. Smith *et al.*, (2000) assessed the soil CH_4 sink to

contribute 10% to the reduction of global CH_4 emissions. Consequently, improved knowledge about the mechanisms behind controls of CH_4 sinks and sources might contribute to increase the reliability of these global estimates and might also support prospective modelling of the CH_4 sinks and sources with ongoing climate change.

The terrestrial CH_4 sink

Soil physical and chemical properties that impact CH_4 uptake vary among ecosystems thereby complicating models estimating the terrestrial CH_4 sink. Smith *et al.* (2000) compared CH_4 net uptake of soils in ecosystems with different vegetation types. Among others, aerated forest and grassland soils which cover 57% of the earth's terrestrial surface area (Constanza *et al.*, 1997) are known to have high CH_4 uptake potentials. In forest soils, CH_4 uptake rates reached up to 25.6 kg CH_4 ha^{-1} yr^{-1}, however, in the majority (70%) of the forest soils CH_4 uptake rates were 0.8-6.4 kg CH_4 ha^{-1} yr^{-1} (Smith *et al.*, 2000). The CH_4 sink of the majority (67%) of grassland soils which were investigated in the same study was 1.6-3.2 kg CH_4 ha^{-1} yr^{-1}, whereas the maximum CH_4 uptake rate was 12.8 kg CH_4 ha^{-1} yr^{-1}. These measurements demonstrate that the CH_4 sink strength varies not only across ecosystems with different vegetation types but also within individual ecosystems.

Methanotrophic bacteria

CH_4 uptake in soils is a strictly biological process, in which microbes (methanotrophic bacteria) in the presence of oxygen assimilate CH_4 and either use CH_4 derived carbon for growth or oxidise CH_4 to CO_2 (Bédard & Knowles, 1989). Since the global warming potential of CH_4 is higher compared to CO_2, not only the CH_4 assimilation is beneficial to reduce global warming but also the oxidation process of CH_4 to CO_2. Methanotrophs are categorized into two different groups, depending on the amount of CH_4 which is required for their growth.

Methanotrophs which require CH_4 concentrations that exceed those in the atmosphere are frequently found in aerated parts of soils, directly above waterlogged soil layers, where CH_4 concentrations are increased due to methanogenic processes (Dunfield, 2007; Rigby *et al.*, 2008). As these methanotrophic bacteria consume CH_4 from deep soil horizons before it reaches the atmosphere, methanotrophs compose a filter function for CH_4 in their inhabiting soil layer. Several *genera* of this type of bacteria are known (Whittenbury *et al.*, 1970; Knief *et al.*, 2003). In contrast, little is known

about methanotrophs consuming CH_4 from the atmosphere. These microbes, which live in aerated upland soils, could not be cultivated until to date (Knief & Dunfield, 2005).

Impacts on the soil CH_4 sink

The CH_4 sink can be influenced by several variables such as soil texture and aggregation (Boeckx *et al.*, 1997; Doerr *et al.*, 1993), the soil water regime (Adamsen & King, 1993; Price *et al.*, 2004), humus or leaf litter (Brumme &Borken, 1999; Gulledge *et al.*, 1997; Dong *et al.*, 1998). Fertilization, especially with ammonium (NH_4^+) based inorganic fertilizers (Willison *et al.*, 1996; Bronson & Mosier 1994) frequently reduce uptake of CH_4 in soils. NH_4^+ is assumed to be either toxic to methanotrophs or reduces CH_4 uptake, since NH_4^+ and CH_4 may serve as competitive substrates for methanotrophs (Whitenbury *et al.,* 1970). Soil pH is an additional parameter involved in the regulation of the soil CH_4 sink (Benstead & King, 2001; O'Neill & Wilkinson, 1977). Soil acidification might not only directly influence methanotrophs (which is still subject of discussion) but acidification clearly impact other processes that might be relevant for the soil CH_4 sink, e.g. nitrification or the release of aluminium (Al^{3+}) ions (as Al^{3+} is toxic to methanotrophic bacteria; Nanba & King, 2000; Tamai *et al.*, 2007). Further knowledge about the variables which severely reduce the soil CH_4 sink as well as the underlying mechanisms is important, since the recovery of methanotrophic bacteria may take more than hundred years (Priemé *et al.*, 1997). Menyailo et al. (2008) found that after the land use change of a pasture to a forest, for which the top 50 cm of the soil was mechanically homogenized, soil CH_4 rates in the 35 years old forest were still three times lower than in the surrounding grassland soil although the species composition appeared to be similar in both soils. Agricultural soil management therefore represents a severe threat for the CH_4 sink of soils. However, negative effects may be avoided, if sufficient knowledge about how the spatial distribution of CH_4 assimilation changes under anthropogenic soil treatments or changing soil parameters becomes available.

Current gaps in knowledge

Although numerous studies exist which describe the influence of various parameters on atmospheric CH_4 uptake in soils, the mechanisms behind these relations are still not fully understood, particularly, as many results are contradictory. For example,

NH_4^+ based fertilizers were in some studies reported to reduce the CH_4 uptake potential of soils (Hütsch *et al.*, 1994; Mosier *et al.*, 1991), while in others fertilization had no effect (Flessa, 1995; De Visscher & Van Cleemput, 2003). A negative effect of the fertilizer mainly appeared when the fertilizer was inorganic despite both, organic and inorganic fertilizers, released NH_4^+ (Willison *et al.*, 1996). It also remains unclear why low soil pH has a negative effect on the CH_4 sink only occasionally (Kasimir-Klemedtsson *et al.*, 1997; Hütsch *et al.*, 1996) and why the depth of the soil water table is not always regulating CH_4 uptake (Adamsen & King, 1993; Guckland *et al.*, 2009).

These contradictory findings might be explained when one knows where in the soil CH_4 uptake is affected by the investigated treatments or soil properties. As physical and chemical properties are heterogeneously distributed in the soil, they might reduce CH_4 assimilation in parts of the soil only. Depending on the fraction of the soil in which CH_4 uptake is inhibited or reduced, the CH_4 sink might differently respond to a treatment. However, approaches to study the spatial distribution of CH_4 assimilation in undisturbed soils at the small scale are still lacking. Little is known about atmospheric CH_4 assimilation in soils by methanotrophs. So far, CH_4 uptake rates of individual soil layers of soil cores were measured in the laboratory (Czepiel *et al.*, 1995; Adamsen & King, 1993; Bender & Conrad, 1994) and CH_4 uptake rates of different soil fractions were determined to assess to which size fraction of the soil grains CH_4 assimilation is mainly attached (Bender & Conrad, 1994). Methods in which soils have been sieved or parts of soils have been separately analyzed were commonly biased by diffusion effects (Roslev *et al.*, 1997). Conclusively, measurements of the CH_4 uptake of soils will be more accurate if performed on intact soil samples. In a different approach, methanotrophs in soil cores were labelled with $^{14}CH_4$ and then the radioactivity was measured in different soil depths (Roslev *et al.*, 1997). Recently developed approaches using ^{13}C labelling of phospholipid fatty acids (PLFA) of methanotrophs (Maxfield et al., 2006) reveal information about the biomass of active methanotrophs in the soil. However, these methods require displacement of parts of the soil. The spatial context between CH_4 assimilation and its spatial origin in the intact soil thereby gets lost.

1.2 Objectives

This thesis focuses on the understanding of atmospheric CH_4 uptake regulation in aerated soils.
The objectives were

i.) to develop a laboratory protocol, which allows studying the spatial distribution of atmospheric CH_4 uptake in intact soils by visualizing $^{14}CH_4$ assimilation

ii.) to investigate the spatial distribution of CH_4 assimilation in relation to the soil depth, soil aggregates, moisture, humus layers, stones, roots, pH, drought and nitrogen fertilization

iii.) to assess the climatic relevance of distinct spatial distributions of CH_4 uptake by analyzing them in combination with CH_4 net flux data.

1.3 Thesis project-overview

The approach applied within this thesis resulted in autoradiographic images that showed the spatial distribution of active methanotrophs in intact soils at the micro-scale. Moreover, information about the relative activity of these microbes involved in CH_4 assimilation was gained. The novelty of the approach consists in the combination of $^{14}CH_4$ labelling and autoradiographic images visualizing CH_4 assimilation. Methodology and the evaluation of different techniques and material are described and discussed in detail in chapter 2.

In the first project, this new approach was applied to study the spatial distribution of CH_4 assimilation in the soil structure with respect to pore channels, aggregates and other features in the soil such as roots, stones or organic layers in several Swiss forest and grassland soils. The relative vertical distribution of the assimilated CH_4 was also studied. At one of the forest sites (*Alptal*), we investigated the effect of increased nitrogen deposition on the spatial distribution of CH_4 uptake and on the CH_4 sink. This was done by obtaining data from plots which were irrigated for more than ten years with local rain water supplemented with ammonium nitrate (NH_4NO_3). To test the effect of NH_4NO_3 on the CH_4 sink, *in situ* CH_4 net flux measurements were

performed biweekly for two continuous years using chamber techniques. Additionally, we chose a forest (*Lägern*) and a grassland site (*Furka*) to study different impacts of natural soil pH on CH_4 uptake. Both sites contained soils on different bedrocks, thereby having distinct pH and these locations did not differ in climate. At the *Lägeren* site, CH_4 uptake rates were measured in the laboratory, while at *Furka*, these data were directly gained in the field (chapter 2).

Chapters 3 and 4 report investigations of short- and long-term applied organic and inorganic nitrogen fertilizers on the spatial distribution and the total uptake of CH_4 in soils. In chapter 3, possible climate change scenarios were further considered in the experimental setup. In general, short-term applications of fertilizers show immediate effects on CH_4 uptake, whereas long-term experiments are of interest since these might more clearly show effects of the investigated parameters. A recovery of inhibited CH_4 uptake is less likely to appear in long-term experiments than in short-term experiments as inhibiting effects might stress or even kill the methanotrophs which are assumed to have a strikingly slow growth rate (Dunfield & Conrad, 2000).
We investigated the impacts of short-term applications of cattle urine, an organic fertilizer, and the inorganic fertilizer NH_4NO_3 in the second year of the respective experiment on two grasslands differing in elevation and management intensity (chapter 3). The two sites are *Alp Weissenstein* (1978 m a.s.l., Switzerland), an alpine grassland with low management intensity and *Früebüel* (982 m a.s.l., Switzerland), a sub-alpine grassland with a higher management intensity. At the two study sites, the impacts of the fertilizers were additionally investigated under simulated summer drought. Reduced precipitation in Switzerland is likely to occur in the future summer months caused by global climate change (Frei *et al.*, 2006). Drought has been simulated with rain-out shelters (Kahmen *et al.*, 2005). Each summer, shelters were placed in the field for approximately two to three months, and soil samples were taken at the end of the second period when shelters were installed. In an additional gradient experiment at *Früebüel*, the effect of the amount of the fertilizer was examined using the same fertilizers as in the drought experiment. This was performed to estimate the impact of urine patches from cattle. Large amounts of nitrogen are released from urine patches into the soil as urea, which is then hydrolysed to $NH_4^+$and therefore urine patches are a potential negative factor for CH_4 uptake. The effect of high amounts of NH_4NO_3 was tested to compare effects of inorganic and organic NH_4^+ releasing

fertilizers. At *Alp Weissentein* and *Früebüel*, CH_4 uptake was measured in the laboratory. In addition, data was used from *in situ* net CH_4 flux measurements at *Alp Weissenstein* and *Früebüel* (Hartmann *et al.,* 2010), to assess the climatic relevance of the observed differences in the spatial distributions of CH_4 uptake in the soils of the plots with the fertilizer and drought treatments.

Long-term applications of organic and inorganic nitrogen fertilizers ($(NH_4)_2SO_4$, $NaNO_3$ and solid manure) were studied, which were annually applied for more than a century to permanent grassland in the oldest ecological experiment in the world: the Park Grass experiment at the *Rothamsted* Research station in the UK (chapter 4). This experiment allowed us to study the effect of distinct pH on the spatial distribution of CH_4 and total CH_4 uptake in combination with the fertilizer treatments or without, as the experiment contained a pH gradient of 4-7, achieved by liming. In this experiment, CH_4 uptake rates were measured directly in the field.

In the synthesis (chapter 5), the most important results of chapters 2-4 are summarised and discussed. Chapter 5 contains furthermore suggestions of combinations of this new approach with other techniques such as micro-computed analysis or quantitative PCR (Polymerase Chain Reaction) for gaining additional information and also applications of this combination of methods that we used to solve other research questions are mentioned.

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Chapter 2

Visualization of the small-scale distribution of methane assimilation in soils

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Chapter 2 is in preparation to be submitted.

Abstract

Soil methanotrophic bacteria assimilate atmospheric methane (CH_4). Soil CH_4 uptake is often found to depend on soil aggregation, soil moisture, soil acidity, and soil ammonium concentrations. To better understand the effect of these variables on CH_4 assimilation and to investigate the underlying mechanisms, it is important to know the spatial distribution of CH_4 uptake in soils differing in soil chemical and physical properties. Here, we present a new approach which allows for the first time to study the spatial distribution of CH_4 assimilation in intact soil cores at the micro-scale. Autoradiographic images were generated from soil sections in which methanotrophs were labelled with $^{14}CH_4$ at atmospheric concentrations. We applied this method to several grassland and forest soils which differed in soil aggregation, pH and bedrock to study possible differences in the spatial distribution of CH_4 assimilation in these soils. Moreover, the effect of increased nitrogen deposition on the spatial distribution

of CH_4 uptake was investigated in a conifer forest site treated with elevated amounts of ammonium nitrate (NH_4NO_3). Net CH_4 fluxes of all soils were measured either *in situ* and/or under laboratory conditions to assess which soil properties might lead to high CH_4 assimilation rates.

Results suggest that $^{14}CH_4$ was irregularly assimilated within the uppermost 10 or 15 cm of soils. Compared to grassland soils, in forest soils the main CH_4 fraction was taken up, also at deeper layers of the soil. The spatial distribution of CH_4 assimilation highly varied with soil aggregation and soil moisture. Increased amounts of CH_4 were assimilated at the borders of aggregates, at the surface of soil pores, within some stones and around limestone rocks. Besides the different distributions of ^{14}C around stones, the label was similarly distributed in soils with distinct pH. Hardly any CH_4 assimilated in humus layers. Roots did not appear to impact spatial distribution of CH_4 uptake. Increased nitrogen deposition slightly reduced the CH_4 sink. However, the treatment did not alter the spatial distribution of CH_4 assimilation.

2.1 Introduction

Soils are estimated to remove 10% of global methane (CH_4) emissions to the atmosphere (Smith *et al.*, 2000). The process of CH_4 uptake in soils is strictly biological. The microorganisms involved in CH_4 uptake are methanotrophic bacteria (Bender & Conrad, 1992; Whalen & Reeburgh, 1990; Bedard & Knowles, 1989). Although CH_4 is eventually converted to carbon dioxide (CO_2), CH_4 uptake by methanotrophs is environmentally beneficial because the global warming potential of CH_4 is 33 times higher than that of CO_2 on a per molecule basis when calculated over a time horizon of 100 years (Shindell *et al.*, 2009).

Forest soils were frequently reported to be stronger sinks for atmospheric CH_4 than grassland soils (Boeckx *et al.*, 1997; Willison *et al.*, 1995a). Despite numerous publications of ecosystem level CH_4 fluxes, the current terrestrial CH_4 sink estimates are associated with large uncertainties (Smith *et al.*, 2000), mainly because the environmental and site factors controlling the distribution and activity of soil methanotrophs are poorly understood and scaling therefore is difficult.

The CH_4 sink is mainly affected by variables regulating CH_4 diffusion into the soil, but also pH and nitrogen deposition influenced the CH_4 sink in certain soils. CH_4 diffusion into the soil is regulated by the size and the spatial arrangement of soil particles, by soil texture, moisture (Doerr *et al.*, 1993; Dunfield, 2007; MacDonald *et al.*, 1996) and humus layers (Bédard & Knowles, 1989; Striegl, 1993). CH_4 diffusion in water is very slow compared to the gaseous phase, and thus CH_4 uptake reduced in the moister parts of soils (Borken & Brumme, 2009; Kruse *et al.*, 1996). Soil acidity might directly or indirectly affect the CH_4 sink as CH_4 uptake rates in acidic soils were frequently increased when compared to alkaline soils (Bradford *et al.*, 2001; Borken & Brumme, 1997; Goulding *et al.*, 1995; Sitaula *et al.*, 2001). Increased nitrogen deposition, especially fertilization with inorganic ammonium (NH_4^+) based fertilizers (Willison *et al.*, 1995b), frequently reduced the CH_4 sink of soils (Mosier *et al.*, 1991; Hütsch *et al.*,1993; Bodelier & Laanbroek, 2004), especially when soils were acidified (chapters 3 and 4; Hartmann *et al.*, 2010).

Estimates of terrestrial CH_4 sinks are further complicated by the fact that variables which affect CH_4 uptake in soils not only vary across different ecosystems but even

within soils at particular sites. The spatial distribution of the methanotrophs in intact soils is to a major extent unknown and little attention has been drawn to the spatial dimension of the mechanisms controlling CH_4 oxidation in soils.

Information about the spatial distribution of CH_4 uptake has so far been gained by measuring CH_4 uptake rates of isolated soil layers or of particle size fractions obtained by various techniques (Bender & Conrad, 1994; Whalen & Reeburgh, 1992). These investigations revealed that CH_4 was mainly assimilated within the top 20 cm of the soil (Gulledge *et al.*, 1997) and that CH_4 uptake rates were highest at the interface of A and B horizon (Adamsen & King, 1993; Whalen & Reeburgh, 1992) at approximately 4-10 cm depth (Schnell & King, 1994; Hütsch, 1998). Bender & Conrad (1994) physically fractionated soil samples and found the highest specific activity, relative to the available particle surface, in the 0.5-2mm fraction they isolated. A general difficulty with these methods is that they heavily alter the physico-chemical environment of the methanotrophs before assessing their activity, which may result in artifacts, especially by altering the availability of CH_4 and O_2 (Roslev *et al.*, 1997). Soil methanotrophs below the soil surface are generally exposed to below-atmospheric CH_4 concentrations, at least in upland soils not harbouring significant amounts of methanogens. Measuring CH_4 uptake in isolated, disturbed fractions exposed to ambient air might well result in higher CH_4 assimilation rates than would be observed in the field. Roslev *et al.* (1997) avoided such artifacts by radiolabelling methanotrophs in intact soil cores with $^{14}CH_4$ and analyzing the depth profile of the assimilated ^{14}C. This research showed that it is important to study the spatial distribution of CH_4 assimilation in undisturbed soils. More recently, approaches using $^{13}CH_4$ isotope probing of phospholipid fatty acids (PLFA) were utilized to quantify methanotrophic biomass and identify the active methanotrophic *taxa* in relation to their spatial origin in soil layers or aggregates (Maxfield *et al.*, 2006).

The aim of this study was to develop a method which allows a spatially resolved measurement of CH_4 assimilation in undisturbed soil structure. The imaging approach we describe here bases on an autoradiographic technique which images $^{14}CH_4$ assimilated in intact soil cores. The spatial resolution obtained (~50µm) is far higher than for the analyses published to date; since large contiguous areas can be scanned in

a piece, the method presented allows the concomitant investigation of CH_4 assimilation at scales ranging from soil horizons to the sub-aggregate scale.

We have applied this technique to a wide range of herbaceous and woody ecosystems differing in terms of vegetation, acidity and parent material, and nitrogen status. Our aims were to (*i*) assess the variability in the distribution of methanotrophic activity across ecosystems, and (*ii*) to study the general spatial distribution of active methanotrophs in relation to soil features such as roots, organic and mineral debris, soil pores and aggregates.

2.2 Materials and Methods

Field sites

We analyzed soils samples which we collected in a number of ecosystems in Switzerland with contrasting properties with respect to vegetation types, soil parent material, nitrogen status, and other factors. Some sites also harbored existing experimental, allowing also to test for effects of the applied treatments.

Alptal

This study site is located in a conifer forest (1200 m a.s.l., 47°03'N, 8°43'E) in the Alptal valley in central Switzerland. The climate is cool and wet (mean annual temperature and precipitation of 6°C and 2300 mm, Tab. 2.1). Soils are gleysols (FAO soil taxonomy) with low permeability (Schleppi *et al.*, 1998). The soil surface is covered by a mor (raw humus) layer on mounds and by an anmoor layer in the depressions (Hagedorn *et al.*, 2001) of 4-5 cm thickness. This site belonged to the NITREX project in which ecosystem responses to manipulated atmospheric N deposition were investigated (Tietema *et al.*, 1998). Starting in 1994, 25 kg N ha^{-1} yr^{-1} are added as NH_4NO_3 to locally collected rain water, which has then been sprinkled over the ground vegetation (Tietema *et al.*, 1998; Xu *et al.*, 2009) in N-enriched plots, whereas water without N amendment has been sprinkled over the control plots. The part of the experiment considered here consists of six replicated plots organised in three blocks; half of the plots receive additional NH_4NO_3 while the other half serves as control. We collected one intact soil core per plot on August 28, 2010, when the water table was at a depth of 40 – 45 cm.

Lägeren

This study site (850 m a.s.l., 47°28'N, 8°22'E) is located in mixed broadleaf/conifer forest on the Lägeren Mountain. The dominant tree species is silver beech (*Fagus sylvatica*). We sampled soil cores in adjacent plots located on different parent material. The first is a rendzina soil (FAO soil taxonomy) on calcareous substrate; the second is a cambisol (FAO soil taxonomy) on sandstone (Tab. 2.1). Further details of this site are given in Rühr & Buchmann (2010).

Furka

This sampling site (2450 m a.s.l., 46°34'N, 8°24'E) is in an alpine grassland above the tree line, near the Furka Pass in the central Swiss Alps. As for Lägeren, we sampled soil cores on adjacent plots differing in parent material.

Alp Weissenstein

This site (1978 m a.s.l. 46°35'N, 9°47'E) is in extensively managed alpine grassland.

Früebüel

(982 m a.s.l. 47°7'N, 8°32'E) is an agricultural grassland site. Both soils lack humus layers (Tab. 2.2). For further details of the latter two sites we refer to Gilgen *et al.*, 2009.

CH_4 in situ net flux measurements

In situ CH_4 net fluxes were measured at *Alptal* and at *Furka* using cylindrical PVC chambers with an internal diameter of 28 cm and 25 cm in height. Gas samples were taken three times during 30 minutes. CH_4 concentrations were measured by gas chromatography (Agilent 6890N gas chromatograph equipped with a FID detector; Agilent, Wilmington (DE), U.S.A.). CH_4 flux rates were calculated by linear regression of CH_4 concentration vs. time.

Soil sampling

Samples were taken by placing polyethylene tubes (5.7 cm external diameter, 16 cm in height) into the soil which were subsequently excavated with a spade. To avoid compaction, the soil core was pre-cut with a knife before sampling. At the forest sites, leaf litter was removed before harvesting the soil cores.

At *Alptal*, samples were taken from four of six replicated blocks at August 28, 2008. At *Lägern*, we took four samples in each, the rendzina soil and in the cambisol at August 20, 2008. At *Furka*, we harvested three samples from soil on calcareous bedrock and three samples from soil on silicate bedrock at August 11, 2008. Three soil samples were taken at each site, *Alp Weissenstein* and *Früebüel*, at August 6 and 18, 2008, respectively.

The samples remained in the tubes, sealed at the bottom with caps, when they were placed in gas-tight 3 L glass jars in the laboratory. Samples remained in the jars for 24 hours to equilibrate to laboratory temperature of 20°C before net CH_4 fluxes were measured.

CH_4 uptake of soil cores

CH_4 uptake of soil cores was measured by analysing gas samples taken from the headspace of the jars containing the samples. At the beginning of the measurement, jars were quickly ventilated and samples were taken every two hours up to six hours after the lids were closed. Gas samples were analogous analyzed to the samples from the *in situ* analysis with gas chromatography (same instrument). CH_4 uptake rates were calculated by linear regression of CH_4 concentration vs. time.

Production of $^{14}CH_4$

Cultures of methanogenic archaea *Methanobacterium thermoautotrophicum* (DSM 1053, (Zeikus & Wolfe, 1972) were used to produce $^{14}CH_4$ out of sodium bicarbonate ($NaH^{14}CO_3$) as described previously by Daniels & Zeikus (1982). Briefly, the methanogens were grown under strictly anaerobic conditions in liquid DSMZ medium 131 (DSMZ GmbH, Braunschweig, Germany) at 65°C, pH 7, with gentle agitation (1.67 Hz). The culture was first exposed to a mixture consisting of 80% (vol/vol) hydrogen (H_2) and 20% (vol/vol) carbon dioxide (CO_2) which had been replaced with 100% (vol/vol) H_2 when $NaH^{14}CO_3$ was added. The by-product carbon monoxide (^{14}CO), which evolves during methanogenesis, was converted to $^{14}CO_2$ with the catalyst Hopcalite TM (Dräger, Lübeck, Germany; Harder, 1997) and all $^{14}CO_2$ was trapped in 1 M sodium hydroxide (NaOH) solution. The purified $^{14}CH_4$ was filled in gas-tight glass flasks which were closed with a septum.

CH_4 labelling of soil samples

After determining CH_4 uptake of the soil cores, methanotrophs in the samples were labelled by injecting $^{14}CH_4$ into the jars with a syringe until concentrations of 5-8 ppm CH_4 were reached. The labelling was performed over a period of 7 d and $^{14}CH_4$ has been added (keeping concentrations < 8ppm) until approximately 100 kBq per soil core were assimilated, which corresponds to ca. 250 Bq (g soil)$^{-1}$. The radioactivity of the added $^{14}CH_4$ was measured by taking gas samples from the headspace of the jars with a syringe and injecting the samples through a septum into an oxidiser (OX 500, Zinsser Analytic GmbH, Frankfurt, Germany) and subsequently analyzing radioactivity with liquid scintillation counting (Packard Tri-Carb 2900TR, PerkinElmer, Schwerzenbach, Switzerland). Each sample was counted three times for 10 minutes.

During the labelling procedure, oxygen concentrations were measured to ensure aerobic conditions within the jars (Agilent 8690N gas chromatograph equipped with a TCD detector). Oxygen (O_2) concentrations were increased, if necessary, by adding O_2 with a syringe to maintain levels of 15-21%. $^{14}CO_2$ evolving from microbial respiration was trapped with 1.5 M NaOH in plastic tubes placed within the jars.

Preparation of soil sections

Soil samples were freeze-dried and embedded in epoxy resin in a vacuum chamber (VTR 5036, Heraeus AG Hanau, Germany) under a negative pressure of 25 kPa during 3 min. The resin consisted of the two components: Laromin C 260 (BASF, Ludwigshafen, Germany) and the hardener Araldite DY 026SP (Astorit AG, Einsiedeln, Switzerland) which were mixed at the ratio 2:3. The resin started hardening after approximately 30 min. The hardening process was accelerated by warming samples to 40-60°C for 2-4 hours 1-2 d after impregnation.

Using a diamond saw (Discoplan, Struers GmbH, Birmensdorf, Switzerland), soil cores were cut into 2-3 horizontal sections of 5 cm height. From each cylindrically shaped section, a slide of 5 cm x 5 cm and 1 cm thickness was cut and fixed on a glass slide (65 x 70 x 3 mm) with epoxy resin. To get plane surfaces of the soil sections, samples were ground with a diamond cup mill (Discoplan, Struers GmbH, Birmensdorf, Switzerland). Applying a vacuum, soil sections were fixed on an adapter which could be moved in parallel to the diamond wheel at a given distance. A

maximum deviation of slide surface planarity <30 µm could be reached. The plane sections were subsequently polished with silicon carbide abrasive paper (grain sizes 600 and 800; Fig. 2.1).

Autoradiography of soil sections

Autoradiographic images of the soil sections were generated by exposing phosphor imaging plates (BAS III S, Fuji Photo Film Co. Ltd., Tokyo, Japan) to the β-radiation of the ^{14}C label which was assimilated at the surface of the slide. Sections were placed on the plates in a light-tight box for 5 days. Using red-excited fluorescence, plates were subsequently scanned at a resolution of 50 µm (Storm 840, Molecular Dynamics GmbH, Krefeld, Germany) which was the highest resolution possible using these plates.

Surface planarity of the samples increased the quality of the autoradiographs, as measurements require close contact of the sample to the plate in order to minimize diffusion of ^{14}C radiation. As radiation of ^{14}C does not reach further than 10 µm within solid material, plane sections and not thin sections were used.

Image processing

With increasing label activity, pixels on the autoradiographic images get darker. As β-decays also occur in the ambient air the background signal of the images increases with prolonged exposure. A background correction of the autoradiographic images of the soil sections was therefore applied. Matrices which contained numbers representing grey shades corresponding to the radioactivity were used for these calculations. A background value was calculated on each side of the slide for each pixel row: We defined areas on the left and right side of each slide, averaged values of each area per row and interpolated a vertical profile line through the averages. We interpolated the values from the left and the right side of the image of the soil sections over the width of the section, thereby generating a background matrix. The background matrix was finally subtracted from the slide matrix. Background corrected matrices of each of the two to three vertical soil sections of a core were then fitted together to one large single matrix per core and values were normalised (i.e. the average value across all pixel values equals one). This later step was necessary as cores were labelled individually. Even small differences in ^{14}C concentrations in the jars might lead to different ^{14}C assimilation intensities, which would no longer allow

to compare distribution patterns among cores. The relative vertical distribution of the ^{14}C label was calculated by averaging the grey values of each pixel row of the background corrected matrix and subsequently normalising these values (i.e. the average values of the profile equals one). Normalised values of every 2.5 cm were averaged. Conventional photographic images were made of each soil section (Coolpix 5400, Nikon, Tokyo, Japan) to identify the occurrence of ^{14}C within the soil and in relation to soil features such as roots or stones.

Statistical analysis

Differences in the vertical ^{14}C distribution among the sites and vegetation types were analyzed with a maximum likelihood approach in R (R 2.8.1, R Development Core Team 2004). In the models, ecosystem or site as well as soil depth were treated as fixed effects and site and replicates as nested random effects. Another model was used to analyze nitrogen effects on the relative vertical ^{14}C distribution at *Alptal:* Nitrogen treatment and soil layer were treated as fixed effects and block and plot were nested random effects. Substrate effects in the vertical ^{14}C distribution at *Lägeren* and *Furka* were analyzed with ANOVA using a model in which ^{14}C assimilation in each layer of 2.5 cm was tested vs. depth and bedrock of the site.

2.3 Results

^{14}C distribution in soil cores

The spatial distribution of CH_4 uptake differed across the study sites (Fig. 2.2). In the forest soils, CH_4 assimilation was increased at the edges of the aggregates while in the grassland soils, ^{14}C was more homogeneously distributed.

Roots did not seem to affect the ^{14}C distribution at *Alp Weissenstein* and *Früebüel*. In the organic layers at *Alptal* and *Furka*, hardly any $^{14}CH_4$ was assimilated. Large differences in the spatial distributions of assimilated $^{14}CH_4$ could be observed within and around certain stones. At *Lägeren*, increased amounts of label were detected around limestone rocks. However, we could not distinguish whether the ^{14}C was assimilated directly at the surface of these rocks or at the surface of soil particles close by (Fig. 2.3a). Increased amounts of ^{14}C have furthermore been assimilated around small pore channels in some of the sandstones at the (Fig. 2.3b) and some of the limestone rocks at *Furka* (Fig. 2.2). Besides increased amounts of assimilated $^{14}CH_4$

around stones, the spatial distribution of CH_4 uptake was similar for the two locations which distinct pH at *Lägeren* and *Furka*. At *Furka*, the main CH_4 assimilating soil layer visually appeared slightly wider at the calcareous than at the silicate location. In general, the CH_4 assimilating soil layer at *Furka* was rather thin compared to the other sites. At *Alptal*, the nitrogen treatment had no effect on the spatial distribution of assimilated $^{14}CH_4$.

Compared to rather sandy soils, fine textured soils containing large fractions of clay and silt might assimilate higher amounts of CH_4 due to lower gas diffusion and higher water retention capacity (Boeckx *et al.*, 1997; Dörr *et al.*, 1993). In soils at *Lägern*, CH_4 uptake was higher at the sampling location with the silt compared to the clay loam; however, we could not observe any difference in the ^{14}C distribution between the samples of the two sites.

The statistical analysis of the relative vertical ^{14}C distributions revealed that the $^{14}CH_4$ was assimilated irregularly within the soil cores of 10 – 15 cm height (P<0.05 at each site). Compared to grassland soil, in forest soils the relative vertical distribution of CH_4 uptake was different (P<0.001): In grassland soils, the main fraction of $^{14}CH_4$ assimilated higher in the soil profile than in forest soils and the cores showed a distinct maximum in CH_4 uptake in 2.5-5.0 cm depth in grassland soils compared to 4-10 cm in forest soils (Fig. 2.4). Moreover, the relative vertical distribution of $^{14}CH_4$ significantly differed among the study sites (P<0.01). In soils on different bedrocks and pH, the relative vertical distribution of assimilated CH_4 was similar at *Lägeren* and at *Furka* and no effect of increased nitrogen deposition was found at *Alptal*.

Net CH_4 uptake of soils and soil water contents

Soils at *Alptal* both emit and consume CH_4. By averaging results of two years of *in situ* measurements (P. Schleppi, unpublished data), both, the untreated and the fertilized soil at *Alptal* were CH_4 sources (13.4±8.1 and 26.0±5.4 μmol CH_4 m^{-2} d^{-1}, respectively, data not shown). When soils oxidized CH_4, average rates during the two years were 18.4±1.4 and 13.9±0.9 μmol CH_4 m^{-2} d^{-1} at the control and fertilized plots, respectively (data not shown). *In situ* net CH_4 fluxes at the day of the soil sampling and soil moisture content of the soil cores from *Alptal* are listed (Tab. 2.2). Laboratory flux measurements of these cores revealed that one sample from the NH_4NO_3

fertilized plots was emitting CH_4. This sample was excluded from spatial analysis of CH_4 uptake as the labelling method requires CH_4 uptake.

Net CH_4 uptake from soil cores at *Lägeren* was in a similar range as in the control plots at *Alptal,* despite the slightly lower soil moisture of the *Lägeren* samples (Tab. 2.2). At the alpine grassland site *Furka,* CH_4 uptake rates measured *in situ* were highest compared to the other sites: Average CH_4 uptake rates of all three measurements were 178.8±12.8 and 123.0±12.4 μmol CH_4 m^{-2} d^{-1} at the silicate and limestone sampling location, respectively. The peak net CH_4 uptake rate at *Furka* was 265 μmol CH_4 m^{-2} d^{-1} and has been measured in soil over silicate bedrock. As at the day of soil sampling, no *in situ* measurements had been made, results from *in situ* measurement which were closest to the soil sampling are listed (Tab. 2.2). These rates were measured 17 d after the soil sampling.

Soil pH did not appear to directly affect the CH_4 sink as at *Furka* CH_4 uptake was higher at the more acidic of the two locations whereas at *Lägeren* opposite results were achieved.

At the high elevated grassland site *Alp Weissenstein*, CH_4 uptake rates were high compared to *Früebüel* (Tab. 2.2). CH_4 fluxes from *Alp Weissenstein* and *Früebüel* samples were both measured in the laboratory and confirmed by *in situ* measurement of CH_4 fluxes at both sites (Hartmann *et al.*, 2010).

2.4 Discussion

Preparation of soil sections

The approach we introduce here resulted in autoradiographic images showing the spatial distribution of CH_4 assimilation in soils. Developing this approach, we considered different media to embed the labelled soil cores. Ideally, the embedding media would possess of the following properties: (*i)* It would mix with water, at least to a certain extent, so that the labelled soil cores would not have to be dried, thus avoiding a spatial rearrangement of soil particles as soils shrink. (*ii)* It would leave soil microbial cells intact, thus not spreading labelled cell constituents during soil impregnation and resin curing. (*iii)* It must have a low viscosity when applied, combined with a suitable settling time; only under these conditions can soil be fully

impregnated. (*iv*) The cured resin needs to be very hard so that the resin core can easily be sawn and ground.

We have considered polyester resins as they are commonly used for soil thin-sections used for soil micromorphological analyses (Beckmann, 1997). These resins have many properties that are ideal to impregnate soils; they have a low viscosity and settle very slowly, therefore infiltrating soil structure very effectively. Finally, the cured resin is very hard and can easily be worked mechanically. However, a serious drawback of these resins is that they are generally dissolved in styrene, which will very effectively lyse microbial cells. Also, the general procedure is to desiccate soils with acetone prior to impregnation (Tippkötter & Ritz, 1996), which would aggravate the situation. Although one could also freeze-dry the soils accepting some disturbance of soil structure, we decided not to use this resin for these reasons.

Instead, we conducted some tests with mixtures of polyethylene glycol (PEG) and polyvinyl alcohols (PVA). These wax-like media are water soluble to some extent. We ran several tests to prepare sections with a freezing microtome. However, the differences in hardness of soil particles and embedding media were too large, so that the surface was heavily disturbed by the microtome and this procedure therefore abandoned. The epoxy resin we finally used proved a suitable compromise between the desired properties. Although the soil impregnation results in some disturbance, these effects are relatively small and the loss in resolution acceptable.

^{14}C activity and label concentration

Results from tests using $^{14}CH_4$ produced from $NaH^{14}CO_3$ with a specific activity of 1480-2220 MBq mmol^{-1} revealed that if soils were labelled with <200 Bq (g soil)$^{-1}$ autoradiographic images were low in contrast. The quality of the images highly improved when activity was increased to >250 Bq (g soil)$^{-1}$. Label assimilation with up to 600 Bq (g soil)$^{-1}$ did not further increase the sensitivity of the imaging approach. As a short labelling time (<2 weeks) is crucial for the specificity of our method, a high specific activity of $^{14}CH_4$ is required to achieve sufficient radioactive label in the soil. A prolonged labelling time might cause errors regarding the spatial distribution of CH_4 uptake, as e.g. protozoa or other organisms could incorporate labelled methanotrophs and transport them away from their origin.

Autoradiographic images

Phosphor imaging plates were scanned at a resolution of 50 µm. However, radioactive decays including background radiation occur randomly. The probability that a signal originated from labelled methanotrophs and not from the ambient air increased with the local pixel number of the signal. This means that observed details in the ^{14}C distribution which are clearly derived from methanotrophic colonies have a resolution that is lower (rather 200-300 than 50 µm).

As soil cores were individually labelled, comparability of ^{14}C distributions between autoradiographs from different samples requires normalization and thus, we analyzed only the relative distribution of the CH_4 assimilation within the single soil cores. Equal grey shades of the different cores can not be interpreted as equal amounts of assimilated CH_4. The development of a labelling device that allows labelling methanotrophs in cores under the same $^{14}CH_4$ atmosphere can improve the present method as quantitative information about the spatial uptake of CH_4 in soils could be compared.

Impacts on CH_4 uptake

The autoradiographic images of the labelled soil sections show differences in the spatial distributions of CH_4 uptake in soils at forest and grassland sites. Lower CH_4 uptake rates were measured in the forest soils compared to the grassland soils, which might be due to the higher soil moisture contents of the forest soils compared to the grassland soils. The soil moisture regime is an important factor controlling CH_4 uptake of soils (Dunfield, 2007). Soil aggregation appears to impact distributions of CH_4 assimilation. The two forest soils had higher contents of soil organic carbon than the grassland soils (Tab. 2.1). High concentrations of soil organic matter was previously shown to improve soil water retention of aggregates (Blanco-Canqui & Lal, 2004; Zhang, 1994). CH_4 diffusion into the centre of aggregates is therefore likely to be limited for most of the time in moist forest soils thereby restricting CH_4 assimilation to the edges of the aggregates. Specific surfaces of soils vary with aggregation, thus atmospheric CH_4 uptake might depend on soil aggregation.

Soils at *Alptal* and *Furka* contain humus layers in which CH_4 uptake is generally low (Dong *et al.*, 1998; Koschorreck & Conrad, 1993). In fact, the autoradiographic images showed little $^{14}CH_4$ uptake in the humus layers of these forest and grassland

soils. Humus layers can reduce atmospheric CH_4 uptake of soils (Dong *et al.*, 1998; Saari *et al.*, 1998; Guckland *et al.*, 2009) by restricting diffusion of CH_4 into the uppermost mineral soil layer because they tend to adsorb high amounts of water (Brumme & Borken, 1999, Dong *et al.*, 1998; Saari *et al.*, 1998). Although the humus layer at *Alptal* is up to 5 cm high (Tab. 2.1), autoradiographic images showed distinct CH_4 assimilation in the uppermost mineral layer. Despite a humus layer of 2 cm thickness, CH_4 uptake rates at *Furka* were still very high (Tab. 2.2), indicating that CH_4 diffusion into the mineral horizon of soils is not severely reduced by humus layers.

$^{14}CH_4$ assimilation occurred within some of the sandstones, limestone rocks and pieces of charcoal. As these differ in chemical properties, we suggest that rather their physical properties might be preferred by the methanotrophs, e.g. pores might protect methanotrophs from negative impacts such as predators. Small pores indeed can provide protection for bacteria from protozoa (Chenu *et al.*, 2001; Vargas & Hattori, 1986). At *Lägern*, $^{14}CH_4$ was assimilated around limestone rocks. We suppose that either the presence of an increased alkaline milieu in the closer surrounding of limestone rocks attracted the methanotrophs or that $^{14}CO_2$ derived from microbial $^{14}CH_4$ respiration was chemically fixed at the surface of the limestone rocks as a part of the calcification process (Schätzl & Anderson, 2005) and thus escaped fixation by the NaOH trap in the labelling jar.

In some forest and grassland soils, applications of NH_4NO_3 reduced the CH_4 sink (Hartmann *et al.*, 2010; Steudler *et al.* 1989; Mosier *et al.*, 1991). Nitrogen deposition at *Alptal* had no effect on the spatial distribution of CH_4 uptake in the soil. In the control plots at *Alptal*, water table height weakly correlated with CH_4 fluxes (R^2 = 0.30), while no correlation could be found in the plots irrigated with NH_4NO_3 (R^2 = 0.06). In the latter case, we suggest a weak negative impact of nitrogen on the CH_4 sink in the fertilized soil. We further suggest that high soil moisture might have prohibited major alterations in the spatial niche of active methanotrophs at *Alptal,* as similar results were found in fertilizer experiments at *Früebüel* (chapter 3; Hartmann *et al.,* 2010). The nitrogen treatment did not acidify soil at *Alptal*. This lack of soil acidification might have reduced the effect of the NH_4NO_3 on CH_4 uptake. Moreover, a part of the NH_4NO_3 was likely absorbed in the distinct humus layer at *Alptal*, in

which CH_4 uptake is naturally low, thereby reducing the amount of NH_4NO_3 which reached the uppermost mineral soil layer where CH_4 uptake was highest in soils of the unfertilized plots.

2.5 Conclusions

The spatial distribution of CH_4 uptake in intact soils can be studied with the help of autoradiographic images derived from soil sections which contain $^{14}CH_4$ labelled microbes and which are embedded in epoxy resin. The autoradiographic images we produced here delivered detailed information at the micro-scale about where in the undisturbed soil increased amounts of atmospheric CH_4 assimilated.

First results of this new approach revealed that the spatial distribution of CH_4 assimilation differs in soils with distinct vegetation and soil structure. For example, in well aggregated soils, CH_4 assimilation was less homogenously distributed than in poorly aggregated soils. Results further indicate that soil moisture further impacts these distributions. Negligible amounts of atmospheric CH_4 assimilate in humus layers of soils. This approach enables to study CH_4 assimilation in relation to soil particles of different chemical composition in great detail, however, this requires additional analysis of the soil.

2.6 Acknowledgements

We thank Leo Meile for providing laboratory facilities, Carla Soliva and Samuel Bucher for help with methanogenic cultures, and Patrick Flütsch, Annina Zihler, Carmen Kunz and Annika Ackermann for technical support. Patrick Spielmann and Roland Wenger are acknowledged for their help with ^{14}C analysis. Frowin Pirovino and Sarah Wernick helped to prepare the soil sections. Adrian Hartmann and Samuel Schmid measured net CH_4 fluxes *in situ*. We also are grateful to Daniel Stiehl for helpful discussions. This work was founded by a Swiss National Science Foundation grant 315230-112681 to PAN. PAN gratefully acknowledges funding by "TUMSS" (Towards the Understanding of Methane Sinks and Sources), a project of ETH's School Domain of Earth, Environment and Natural Resources. This project was conceived by PAN, PS measured net CH_4 fluxes *in situ* at *Alptal*, the laboratory techniques were developed by PAB with input by PAN and EK and applied by PAB, and writing was done by PAB and PAN with input from the other co-authors.

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2.8 Tables and Figures

Table 2.1: Overview of the characteristics of the main study sites *Alptal*, *Lägeren*, *Furka*, *Alp Weissenstein* and *Früebüel.*

	Alptal		*Lägeren*		*Furka*		*Alp Weissenstein*	*Früebüel*
Treatment	Control	NH_4NO_3	Control		Control		Control	Control
Elevation (m a.s.l.)	1200		850		2450		1978	982
Mean annual Temperature (°C)	6.0 [1]		8.4 [5]		-2.1 [5]		2.0 [7]	7.5 [7]
Annual Precipitation (mm)	2300 [1]		930 [5]		1920 [5]		920[7]	1630[7]
Vegetation	Coniferous forest [2]: Predominantly norway spruce (*Picea abies*) with 15 % silver fur (*Abies alba*)		Mixed mountain forest [5]: Predominantly beech (*Fagus sylvatica*), spruce (*Picea abies*) and maple (*Acer pseudoplatanus*)		Alpine grassland		Alpine grassland	Subalpine grassland
Bedrock	Flysch sandstone with argillite and bentonite shists [2]		Limestone	Sandstone	Limestone	Silicate	Moraines and Calcareous shists	Molasse
Soil type	Clayey gleysol [2] [6]		Rendzina [6]	Cambisol[6]	-	-	Regosol	Gleyic cambisol[6]
Soil texture	Silty clay [2]		Silt	Clay loam [b]	Silt	Silt loam	Silt loam	Silt loam
Bulk density (g cm^{-3})	0.37 ± 0.07		1.02 ± 0.10	1.32 ± 0.10	1.08 ± 0.01	1.13 ± 0.11	0.93 ± 0.02	1.12 ±0.03
Soil pH	3.8	3.8	6.7	5.8	6.5	4.2	5.0	4.7
C (%)	25 [1]	18 [1]	13 [4]	4 [4]	4	4	7	3
Humus layer (cm)	4 - 5	1 - 5	0	0	1 - 3	0 - 2	0	0

(1) Xu *et al.*, 2009
(2) Hagedorn *et al.*, 2000
(3) Rühr & Buchmann, 2010
(4) Wehrli, 2006
(5) Hurni (ed.), Atlas of Switzerland, version 2.0.1, Swisstopo,
(6) FAO accepted guidelines for soil description, 1990
(7) Zeeman *et al.*, 2010

Table 2.2: Soil CH_4 uptake rates (µmol m^{-2} d^{-1}) and soil moisture (m^3 H_2O cm^{-3}) from soil at the sampling sites *Alptal, Lägeren*, *Furka*, *Alp Weissenstein* and *Früebüel* at the day of soil sampling if not mentioned otherwise in the text (mean values ± standard errors).

Site	Treatment / Bedrock	CH_4 uptake (µmol m^{-2} d^{-1})	Soil moisture (vol. % H_2O)
Alptal	Control	10.8 ± 19.8	60.2 ± 3.6
	NH_4NO_3	-8.0 ± 12.9	56.4 ± 15.7
Lägeren	Limestone	14.0 ± 0.3	41.0 ± 2.4
	Sandstone	8.0 ± 1.0	43.5 ± 3.3
Furka	Limestone	80.4 ± 10.0	27.3 ± 2.0
	Silicate	163.5 ± 16.9	31.8 ± 3.5
Alp Weissenstein	Control	62.6 ± 12.4	43.1 ± 5.1
Früebüel	Control	23.8 ± 0.2	49.0 ± 6.5

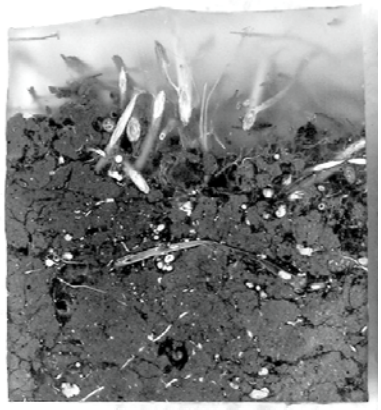

Figure 2.1: One of the soil sections (5 x 5 cm) of which autoradiographic images were made of. The resin consists of Laromin C 260 and Araldite DY026 SP mixed at a weight ratio of 2:3. Sample is sawn with a circular saw and ground with a diamond cup mill.

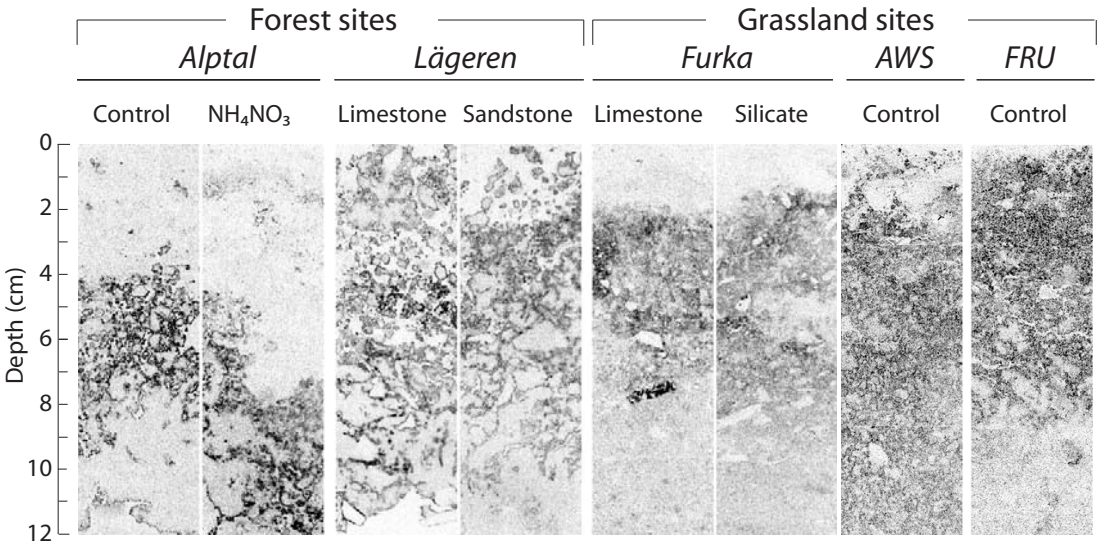

Figure 2.2: Autoradiographic images showing CH_4 distribution in the top 12 cm of one out of three or four replicated soil cores of the sampling locations *Alptal*, *Lägeren*, *Furka*, *Alp Weissenstein (AWS)*, and *Früebüel (FRU)*, labelled with $^{14}CH_4$. The images show the relative ^{14}C distribution. Data are standardised, i.e. all pixel values average 1.0. Darker pixels indicate more assimilated ^{14}C.

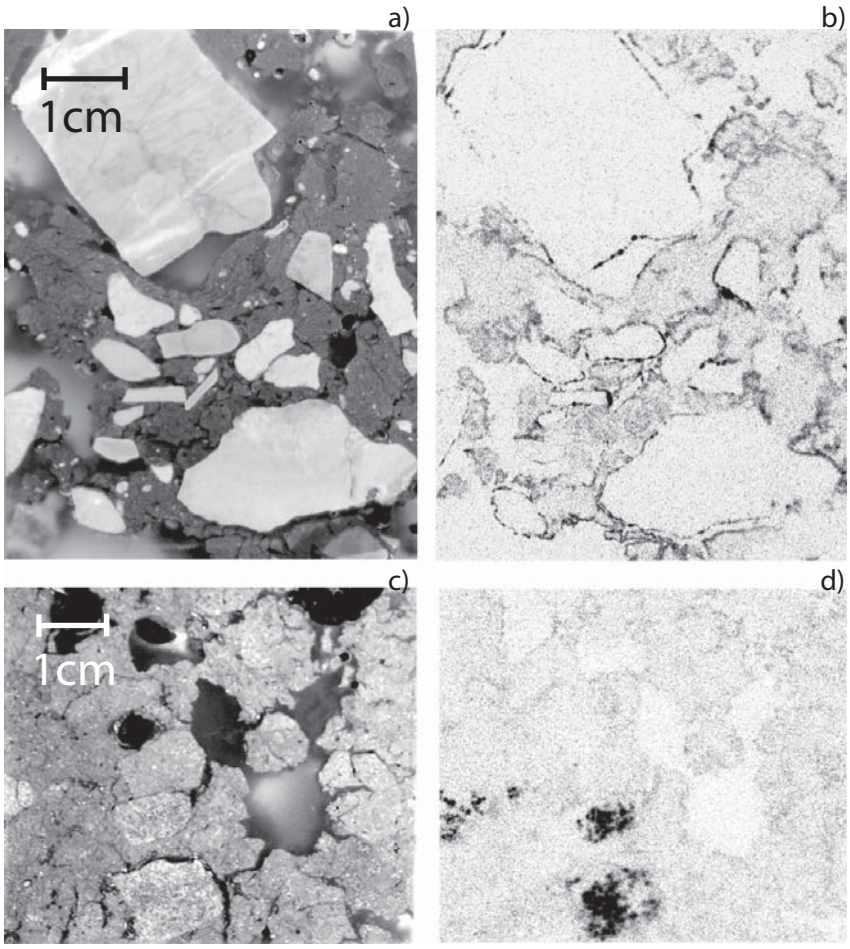

Figure 2.3a: Conventional photographic image (a) and autoradiographic image (b) of a slide from the calcareous sampling location at *Lägeren*. The slide represents soil from approximately 6-12 cm depth. The images show limestone rocks (photography) and increased ^{14}C assimilation around them (autoradiography). Data from the autoradiographic image are standardised, i.e. all pixel values average 1.0, and therefore only show the relative label distribution. Darker grey pixels mean increased label assimilation.

Figure 2.3b: Conventional photographic image (c) and autoradiographic image (d) of a slide from the cambisol sampling location at *Lägeren*. The slide represents soil from approximately 5-10 cm depth. The images show sandstones (photography) and the increased ^{14}C assimilation within the pores of them (autoradiography). Data from the autoradiographic image are standardised, i.e. all pixel values average 1.0, and therefore only show the relative label distribution. Darker grey pixels mean increased label assimilation.

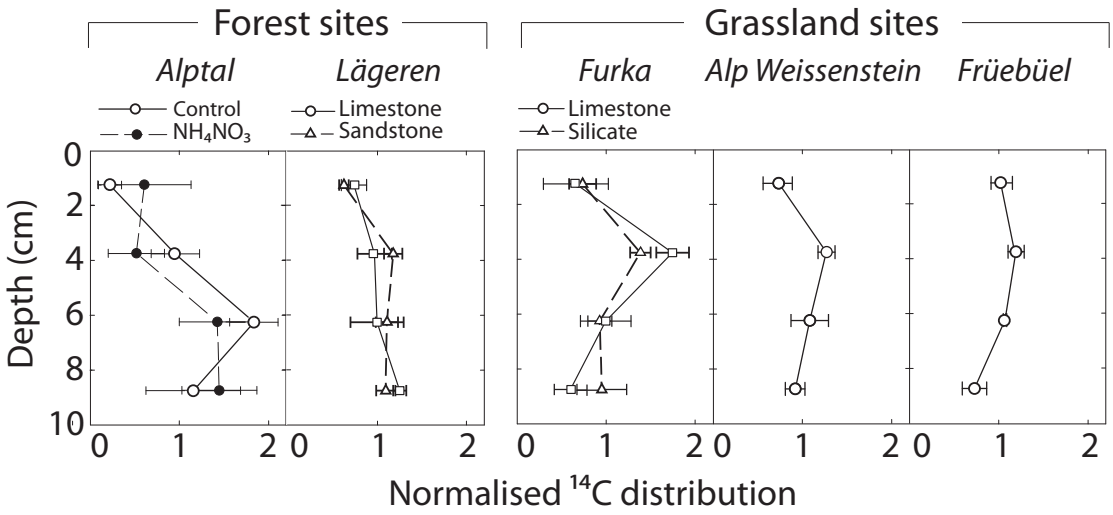

Figure 2.4: Relative vertical ^{14}C distribution of soil cores labelled with $^{14}CH_4$ at *Alptal*, *Lägeren, Furka, Alp Weissenstein* and *Früebüel.* Data are standardised per core (averaged labelling intensity of all layers equals one).

Chapter 3

Interactive effects of drought and N fertilization on the spatial distribution of methane assimilation in grassland soils

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Chapter 3 was submitted 26 May, 2010.

Abstract

Soil methanotrophic bacteria constitute the only globally relevant biological sink for atmospheric methane (CH_4). Nitrogen (N) fertilizers as well as soil moisture regime affect the activity of these organisms, but the mechanisms involved are not well understood to date. In particular, virtually nothing is known about the spatial distribution of soil methanotrophs within soil structure and how this regulates CH_4 fluxes at the soil ecosystem scale.

We studied the spatial distribution of CH_4 assimilation and its response to a factorial drought × N fertilizer treatment in a three-year experiment replicated in two grasslands differing in management intensity. Intact soil cores were labelled with $^{14}CH_4$ and methanotrophic activity mapped at a resolution of 50 µm using an autoradiographic technique. Under drought, the main zone of CH_4 assimilation shifted down the soil profile. Ammonium nitrate (NH_4NO_3) and cattle urine reduced CH_4

assimilation in the top soil, but only when applied under drought, presumably because NH_4^+ from fertilizers was not removed by plant uptake and nitrification under these conditions. Soil CH_4 fluxes measured in the field did show no or only very small inhibitory effects, suggesting that deeper soil layers fully compensated for the reduction in top soil CH_4 assimilation. Our results indicate that the soil CH_4 sink cannot be inferred from measurements on isolated soil samples, because the spatial organization of soils ought to be considered (e.g. stratification of organisms, processes and mechanisms). The autoradiographic technique we have developed is suited to study methanotrophic activity in a relevant spatial context and does not rely on the genetic identity of the soil bacterial communities involved, thus ideally complementing DNA-based approaches.

3.1 Introduction

Atmospheric methane (CH_4) concentrations have more than doubled since preindustrial times, with current concentrations around 1.8 ppm (IPCC, 2001). While many sources exist, both natural and anthropogenic, only two processes remove significant amounts of CH_4 from the atmosphere. Chemical oxidation in the atmosphere constitutes the dominant sink for CH_4, but atmospheric CH_4 is also oxidized by terrestrial soils, a process essentially driven by soil methanotrophic bacteria (reviewed in Dunfield, 2007; Le Mer & Roger, 2001). However, the available estimates of the global soil CH_4 sink are associated with large uncertainties, on one hand because direct field measurements of soil CH_4 fluxes are scarce, on the other hand because the ecology of methanotrophic bacteria of well-drained oxic upland soils is only poorly understood to date, complicating the modelling and scaling of soil fluxes.

A major factor limiting the soil sink for atmospheric CH_4 is restricted diffusion of CH_4 through soils (Striegl, 1993). Soil gas diffusive conductance depends on soil texture, and many estimates of the soil CH_4 sink (including the one in IPCC, 2007) depend on sink parameterizations in dependence of soil texture (Boeckx *et al.*, 1997; Dörr *et al.*, 1993). At any given site, the temporal variability of soil CH_4 uptake is generally well predicted by soil moisture (Adamsen & King, 1993; Price *et al.*, 2004). Soil water limits soil gas diffusion into aggregates by blocking small pore channels and by flooding macro-pore networks (Young & Ritz, 2000), and moisture therefore generally decreases soil CH_4 uptake.

A second important factor controlling soil CH_4 oxidation is nitrogen (N) fertilization. Ammonium-based (NH_4^+) fertilizers frequently reduce soil CH_4 uptake (Bronson & Mosier, 1994; Dunfield & Knowles, 1995; Willison *et al.,* 1995). These observations have been related to the finding that ammonia can competitively inhibit CH_4 oxidation by methanotrophs (Gulledge & Schimel, 1998; Whittenbury *et al.*, 1970). However, the mechanisms underlying N fertilizer-effects are still not fully understood. For example, Steudler *et al.* (1989) found immediate reductions in soil CH_4 uptake upon application of NH_4NO_3 to forest soils, whereas Gulledge *et al.* (1997) reported only strongly delayed effects of N application in taiga forest soils. Also, long-term effects of N fertilizer applications were reported (e.g. Hütsch, 1996; Hütsch *et al.*, 1994;

Mosier *et al.*, 1991), but observations are controversial, with effects depending on soil pH and the chemical form in which N was applied. In pasture, grazing animals redistribute plant N in urine and dung patches (Williams & Haynes, 1994). The N deposition rates in these patches can be extremely high, exceeding several thousand kg N ha^{-1} (Külling *et al.*, 2002). How the soil CH_4 sink responds at the patch level to these impacts is not well understood so far. Adverse effects could especially be expected for urine-N, which quickly hydrolyses to NH_4^+.

It appears that mechanisms vary with the system studied, or that they interact strongly with factors that have not received sufficient attention to date. In this context, a particularly important question is which spatial niche soil microbes involved in CH_4 oxidation occupy. However, virtually nothing is known about the small-scale distribution of methanotrophs in soils.

Soil CH_4 fluxes have mainly been investigated at the whole soil level (in the field) or at the bulk soil level (in the laboratory), thus largely ignoring soil structure and the spatial organization of the involved processes within this structure. Notable exceptions are studies in which CH_4 oxidation or assimilation were analyzed by soil layer (e.g. Roslev *et al.*, 1997; Adamsen & King, 1993; Czepiel *et al.*, 1995; Schnell & King, 1994). However, this approach has limitations, especially if soils were sieved (Roslev *et al.*, 1997). Any disturbance of soil structure will result in a change in the environmental conditions experienced by methanotrophic bacteria, including altered CH_4 concentrations, so that it is unclear whether such measurements actually reflect the natural process rates.

Here, we present a novel technique which allows to study the spatial distribution of active methanotrophic microorganisms in undisturbed soil. In brief, intact soil cores were exposed to $^{14}CH_4$, selectively labelling microbes assimilating CH_4 under atmospheric concentrations. In combination with autoradiographic and soil micromorphological techniques, we were able to map the spatial distribution of CH_4-assimilating microbes in the intact soil structure at a resolution of 50 µm. We used this technique to study the mechanisms underlying ecosystem level effects of N application on soil CH_4 uptake in two grasslands differing in management intensity. N was applied in the form of NH_4NO_3 and cattle urine. The N application treatment was factorially combined with a summer drought treatment, simulating drought episodes

as they are predicted to increase in frequency in Central Europe in this century (Frei *et al*., 2006; Schär *et al*., 2004). In a separate experiment, we varied the amounts of NH_4NO_3 and urine added over a wide range, which allowed us to study how fertilizer effects depend on N application rates. The specific aims of the present study were (1) to localize the site of atmospheric CH_4 oxidation within intact soil cores, (2) to assess whether the spatial distribution of CH_4 assimilation in soils changes under N fertilization and drought, and (3) to establish a link between effects on CH_4 fluxes and associated shifts in the spatial distribution of the underlying methanotrophic activity.

3.2 Materials and Methods

Field site and experimental design

A factorial drought × N fertilizer experiment was replicated at two grassland sites that contrast in elevation and management intensity. The low-altitude site *Früebüel* (982 m a.s.l., 8°32'16''E, 47°6'57''N) is an agricultural grassland east of Lake Zug, Switzerland. The site has been grazed or mown for at least 60 years. Fertilizer input is by animals grazing on the site, and solid manure and liquid cattle slurry from the animals kept in the stable. Mean annual precipitation at this site averages around 1600 mm, with mean annual temperatures between 7 and 8°C (Zeeman *et al.*, 2010). The soil is a silt loam (37% sand, 56% silt, 7% clay) with a bulk density of 1.12±0.03 g cm^{-3} and a total soil organic C content of (4.4±0.2)%, (2.8±0.1)%, and (2.4±0.2)% in the 0-5, 5-10, and 10-15 cm layers, respectively. The top 11 cm show a high root density, and are well aggregated except for the topmost 4 cm. Deeper soil horizons are rich in gravel and stones.

The high-altitude site *Alp Weissenstein* (1978 m a. s. l., 9°47'25'' E, 46°34'59'' N) is an alpine grassland close to the Albula Pass in Graubünden, Switzerland. It has been used as an extensively managed pasture grazed by cattle and horse for the last decades with no additional fertilizer application. Annual precipitation at this site averages around 900 mm, with mean annual temperatures around 2°C (Zeeman *et al*., 2010). The silt loam soil (35% sand, 59% silt, 6% clay) has a pH of 4.5–5.5 and a bulk density of 0.93±0.02 g cm^{-3}. Soil organic C is (11.2±0.3)%, (5.6±0.2)%, (3.9±0.2)% in the 0–5 cm, 5–10 cm and 10–15 cm layer, respectively. The top layer consists of a

2 cm organic mat containing only few soil aggregates followed by a single, poorly aggregated horizon containing very few stones.

The drought × N fertilization experiment was laid out as blocked randomized split-plot experiment. In May 2007, ten large plots (3 m × 3.5 m, organized in 5 blocks) were established at each field site. These plots were subdivided into 4 subplots, resulting in a total of 40 subplots per site. The drought treatment was applied at the plot level, while the N fertilization treatment was applied at the subplot-level.

On half of the plots, transparent rain-out shelters (Kahmen *et al.,* 2005) were installed for six weeks to simulate summer droughts associated with climate change (*Früebüel*: August 3-September 27 in 2007 and June 26-August 13 in 2008; *Alp Weissenstein*: July 31-September 25 in 2007 and July 14-September 26 in 2008).

The N fertilizer treatment consisted of subplots receiving no fertilizer ("NIL"), ammonium nitrate ("NH_4NO_3"), or cattle urine ("urine"). A fourth subplot was treated with cattle dung, but at a much lower application rate, and is not part of the present study. Cattle urine N consists primarily of urea, which hydrolyses to NH_4^+ in soils. We applied urine at half the application rate of NH_4NO_3 on a total N basis, which resulted in the same amount of NH_4^+ being released to soils. For the sake of simplicity, N application rates are reported on the basis of NH_4^+ equivalents in this paper. In 2007, N fertilizer was applied in two portions. First, we applied 50 kg NH_4-N ha^{-1} to plots on August 9 and 10, followed by 150 kg NH_4-N ha^{-1} on September 19 and 24 at *Früebüel* and *Alp Weissenstein*, respectively. This procedure was chosen because we had no *a priori* knowledge of the impact of the N applied and did not want to risk to completely knock out the soil CH_4 sink. In 2008, fertilizer N was applied as a single 150 kg NH_4-N ha^{-1} addition on July 9 and 29 at *Früebüel* and *Alp Weissenstein*, respectively. All fertilizers were applied during the drought simulation period as 3 L aq solution m^{-2}. Plots were then watered with 2 L H_2O m^{-2} to ensure that all fertilizer was flushed into the ground. We refer to Hartmann *et al.* (2010) for further details of site, experimental design, and management.

At *Früebüel*, an additional experiment testing dose-effect relationships of the different fertilizers (but not in combination with drought) was set up in 2007. The experiment consisted of plots receiving the same N fertilizers as in the drought × N fertilization

experiment (NH_4NO_3 and cattle urine) at rates of 0, 20, 60, 180, 540 and 1620 kg NH_4-N equivalents ha^{-1} yr^{-1} (one replicate per fertilizer type and application rate, except for the unfertilized plots which were replicated twice). This experiment allowed to test effects of very high N applications as they can occur in cattle urine patches (>500 kg N ha^{-1}; Külling *et al.*, 2002).

$^{14}CH_4$ preparation

$^{14}CH_4$ was produced following the method of Daniels and Zeikus (1982). In essence, $NaH^{14}CO_3$ with a specific activity of 1.48–2.22 GBq $mmol^{-1}$ (Perkin Elmer, Schwerzenbach, Switzerland) was converted to $^{14}CH_4$ by the methanogenic archaea *Methanobacterium thermoautotrophicum* (DSM 1053; Zeikus & Wolfe, 1972) grown under strictly anaerobic conditions at 65°C in DSMZ medium 131 (DSMZ GmbH, Braunschweig, Germany). $^{14}CH_4$ was purified with Hopcalite™ (Dräger, Lübeck, Germany) that removes ^{14}CO, a byproduct formed in traces by the methanogens that we used (Harder, 1997); $^{14}CO_2$ was removed with sodium hydroxide solution (chapter 2).

Soil sampling

Due to the laborious experimental protocols adopted, the analyses presented here were carried out in three out of the five replicate blocks of the drought x N experiment only. Intact soil cores were collected on August 18, 2008 at *Früebüel* (53 d after the rain shelters had been installed and 40 d after fertilizer application) and on August 6, 2008 at *Alp Weissenstein* (23 d after the rain shelters had been installed and 8 days after fertilizer application). Soils from the separate fertilizer gradient experiment were sampled on September 2, 2008, 55 d after fertilizer application.

The soil cores were collected by pre-cutting soils with a knife and carefully driving polyethylene tubes of 16 cm length and 5.7 cm internal diameter into the soil. The tubes were then excavated from the side with the help of a spade. This procedure minimised soil disturbance and, unlike regular soil coring methods, caused practically no compaction, as was evident from the unchanged length of the harvested soil cores. The polyethylene tubes containing the cores were capped at the bottom and placed vertically in gas-tight 3 L glass jars and equilibrated for 24 h to laboratory temperature (20°C).

CH_4 oxidation rates of soil cores

To measure CH_4 oxidation rates of the equilibrated soil cores, the jars were quickly ventilated, closed again, and CH_4 concentration in the headspace measured every 3 h for a 12 h period (Agilent 6890N gas chromatograph equipped with a FID detector; Agilent, Wilmington, Delaware, U.S.A.). CH_4 oxidation rates were calculated by linear regression of measured CH_4 concentration vs. time. We also monitored oxygen consumption to ensure aerobic conditions in the jar (Agilent 6890N gas chromatograph equipped with a TCD detector).

$^{14}CH_4$ labelling of cores

After having measured their CH_4 oxidation rate, all soil cores were labelled with $^{14}CH_4$ over a period of 7 d. During labelling, $^{14}CH_4$ was added to the jar with a syringe. CH_4 concentrations in the headspace were maintained in the range of 5-8 µL CH_4 L^{-1}. $^{14}CH_4$ addition was continued until a total activity of 100 kBq had been assimilated. This ensured approximately equal labelling of all cores, regardless of their respective CH_4 assimilation rate. $^{14}CO_2$ and unlabelled $^{12}CO_2$ evolving from microbial respiration were trapped in plastic tubes filled with 1.5 M NaOH. O_2 consumption was monitored and kept in the range of 15-20% by adding O_2 with a syringe.

Preparation of soil sections

After ^{14}C-labelling, the soil samples were freeze-dried and impregnated with epoxy resin (Laromin C 260, BASF, Ludwigshafen, Germany, mixed at a ratio of 2:3 with Araldite DY 026SP hardener, Astorit AG, Einsiedeln, Switzerland). To accelerate the infiltration of the soil with resin, the cores were placed in a vacuum chamber (VTR 5036, Heraeus AG, Hanau, Germany) and evacuated to an end pressure of 25 kPa. After 3 min, the samples were slowly brought back to atmospheric pressure and the epoxy resin left hardening for at least 4 days. From field harvesting until impregnation, the soil cores remained upright in their original sampling tube.

The resin cores were then cut horizontally into 3 sections approximately 5 cm in height (Discoplan diamond circular saw, Struers GmbH, Birmensdorf, Switzerland). From each of these cylindrical sections, a vertical slide of 5 cm x 5 cm area and 1 cm thickness was cut and mounted with epoxy resin onto a 3 mm thick glass slide. The surface of these soil sections was then levelled with a diamond cup mill (Discoplan,

Struers GmbH, Birmensdorf, Switzerland). The maximum deviation from surface planarity remaining after this procedure was below 30 µm. The surface of the soil sections was then manually polished with silicon carbide abrasive paper (grain sizes 600 and 800).

Autoradiography of soil sections

The polished soil sections were placed on phosphor imaging plates (BAS III S, Fuji Photo Film Co. Ltd., Tokyo, Japan) which were exposed for 10 d to the ^{14}C radiation emitted from the section's surface. The imaging plates were subsequently digitized by red-excited fluorescence scanning at a resolution of 50 µm (Storm 840, Molecular Dynamics GmbH, Krefeld, Germany). The three 5 cm × 5 cm slides per soil core were re-composed to form a single black and white image, in which darker pixels correspond to more intensive labelling (in analogy to conventional X-ray films). This generally went well, because very little material had been removed by the circular saw. The data processed effectively were data matrices, which can be visualized as bitmap image composed of individual "grey values".

Image processing

β-particles emitted by the ^{14}C bound in the samples do not travel further than ca. 10 µm in the resin matrix, so that the images acquired show a well-resolved two-dimensional distribution of the label at the sample surface. To identify specific soil features (roots, stones etc.), all soil sections were photographed with a conventional digital camera (Coolpix 5400, Nikon, Tokyo, Japan).

The vertical distribution of ^{14}C in the soil profile was quantified by averaging the grey values of the autoradiographic image per horizontal pixel line, corresponding to a vertical resolution of 50 µm. The grey values obtained are averages over all features of the soil profile (soil particles, inter-aggregate voids, small stones, roots, etc.). Although the visual inspection of the scans did not reveal obvious artifacts at the borders of the core (edge effects from labelling and impregnation with the resin), a 1 cm wide strip was excluded from the left and right side of the scan image as a precautionary measure. The imaging plates also accumulate cosmic radiation and background radiation emitted from radioisotopes in the resin, building walls etc., which is significant given the long exposure time and low activity in our samples. Therefore, background grey values were determined from the area left and right of the

image and subtracted from the values in the area exposed by the soil section. Finally, the vertical profile was normalised (i.e. the average data across the profile scaled to 1.0).

The small-scale distribution of CH_4 assimilation within given soil layers was analyzed by generating histograms of the data found in 4 cm wide stripes at 1.5-2.5 cm, 6.0-7.0 cm and 10.5-11.5 cm depth, respectively. The data in these stripes were first corrected for background radiation as described above and the resulting data matrix normalised to 1.0, thus eliminating any effects of potentially different vertical distributions among treatments. The autoradiographic images contained substantial random noise due to the relatively low amounts of ^{14}C that we had applied. All images were therefore smoothed by replacing each pixel with the average value of the surrounding 5×5 pixel square. The frequency distribution of the labelling intensities was then determined by generating histograms with seven intensity classes (<0.4, 0.4-0.8, 0.8-1.2, 1.2-1.6, 1.6-2.0, 2.0-2.4, and >2.4). The frequency distribution was then compared among the experimental treatments.

Statistical analysis

All results were analyzed using models fitted by maximum likelihood as implemented in the *lme* procedure in R (R 2.8.1, R Development Core Team 2009). Field site, block, plot and subplot were treated as nested random effects, while drought, fertilizer, and soil layer were fixed effects.

Effects of NH_4NO_3 and urine on the vertical distribution of ^{14}C were tested by comparing models that either distinguished all three levels of the fertilizer factor (i.e. NIL, NH_4NO_3 and urine) or that lumped together the NIL with the respective treatment. These nested models were then compared with likelihood ratio tests; a difference between models indicates that the respective treatment is significantly different from NIL (main effect or one of the interactions).

Effects of N fertilizers and drought on the small-scale distribution were tested by the interactions of histogram class with the respective treatment factor; for example, a significant drought × histogram class interaction would indicate that the distribution of grey values differed between drought treatments.

3.3 Results

Soil water content

The soil water content of field-moist soil cores averaged 47.6±2.2 and 37.7±1.8 cm^3 H_2O cm^{-3} in the unsheltered plots at *Früebüel* and *Alp Weissenstein*, respectively. The rain exclusion roofs significantly reduced soil moisture at both sites (P<0.001 23.0±2.4 and 22.8±1.1 cm^3 H_2O cm^{-3} in drought-treated plots at *Früebüel* and *Alp Weissenstein*, respectively). Fertilizer treatments did not affect soil moisture.

The soil water content of the cores sampled two weeks later in the separate experiment investigating dose-effect relationships of the applied fertilizers averaged 36.5±0.7 cm^3 H_2O cm^{-3}, with no significant treatment differences.

CH_4 uptake of soil cores

Soil cores collected at *Früebüel* oxidised less atmospheric CH_4 than cores from *Alp Weissenstein* (P<0.01; Fig. 3.1). The drought treatment increased CH_4 uptake of the soil cores at the two sites (P<0.05; Fig. 3.1). Both N fertilizers reduced CH_4 uptake rates (P<0.01). We did not detect a significant site × N interaction, indicating that the the N-effect did not differ significantly between sites.

In the experiment testing dose-effect relationships at *Früebüel,* plots amended with NH_4NO_3 equivalent to 1080 kg NH_4^+-N ha^{-1} or more did not show any significant CH_4 uptake. The oxidation rates of the remaining cores averaged 31.1±1.1 µmol CH_4 m^{-2} d^{-1} (data not shown). An analysis of covariance showed that the N additions reduced CH_4 oxidation (P=0.01), and that this effect was fertilizer-dependent (P=0.05 for the fertilizer type × N amount interaction), but this effect was entirely driven by the two NH_4NO_3 fertilized plots where CH_4 uptake was completely inhibited.

Distribution of assimilated $^{14}CH_4$

The assimilated ^{14}C detected in the autoradiographc images was not homogeneously distributed (Figs. 3.2 and 3.3). Visual inspection showed a concentration of ^{14}C label on the surface of soil aggregates. This was most evident in the soil layers that were well aggregated (at *Früebüel*: below ca. 4 cm), and in soils that were not exposed to drought. There was no evident relation between ^{14}C label and soil features such as plant roots, litter particles or earthworm burrows. There was a tendency for increased

labelling along the surfaces of small pore channels, especially in deeper soil layers to which CH_4 probably did not diffuse easily otherwise.
The assimilated ^{14}C label showed a distinct vertical profile that depended on the applied N and drought treatments, but it also was very variable at the small scale, reflecting soil structure (Fig. 3.3). It should be emphasised that the data presented here show standardised $^{14}CH_4$ assimilation patterns (because approximately equal amounts of $^{14}CH_4$ had been applied to each core). For example, a decrease in ^{14}C labelling in the top soil can indicate a lower methanotrophic activity in this layer, but it can also be the result of a stronger relative increase in $^{14}CH_4$ assimilation in deeper layers.

Simulated drought increased $^{14}CH_4$ assimilation in deeper soil layers (Fig. 3.3, $P<0.001$ for the drought × layer interaction), and this effect differed between sites ($P<0.001$ for drought × site × layer). Both urine and NH_4NO_3 significantly decreased the assimilation of CH_4 in top soil layers ($P<0.05$ for fertilizer × layer interaction). However, at *Früebüel*, the fertilizer effect was more pronounced in the drought treatment, while this was not the case at *Alp Weissenstein* ($P<0.05$ for fertilizer × drought × layer interaction at *Früebüel* but not significant at *Alp Weissenstein*; sites analyzed separately).

We analyzed the spatial distribution of assimilated ^{14}C within each soil layer by comparing the frequency distribution of grey values in the autoradiographic images. The distribution of grey values was site and layer-specific ($P<0.001$ for site, layer, and $P<0.01$ for site × layer, Fig. 3.4). Simulated drought altered the spatial ^{14}C assimilation patterns ($P<0.01$). This effect depended on site ($P<0.001$ for site × drought) and decreased with depth ($P<0.001$ for drought × layer interaction). The most important effect found was that N fertilizer application led to a concentration of ^{14}C assimilation in a smaller area of the section surface ($P<0.001$). This is evident from the higher frequency of pixels with very low grey value, indicating large areas with no CH_4 uptake (Fig. 3.4). The N fertilizer effect decreased with depth ($P<0.001$).

Plots of the fertilizer gradient experiment at *Früebüel* were never covered by rain shelters and are therefore comparable with control plots from the main experiment at *Früebüel* in terms of soil moisture dynamics. Urine had only a relatively minor effect on the $^{14}CH_4$ distribution in soil cores (Fig. 3.5). The autoradiographic images may

show a slight inhibition of $^{14}CH_4$ assimilation in the top 1-2 cm at the highest urine application rate of 1620 kg N ha^{-1}, but this cannot be substantiated in statistical terms due to a lack of replicates. In contrast, NH_4NO_3 completely inhibited $^{14}CH_4$ assimilation at application rates equal or greater than 540 kg NH_4-N ha^{-1}. The analysis of the small-scale distribution of ^{14}C showed that the application of high amounts of NH_4NO_3, and to a much lesser extent also of urine, progressively restricted $^{14}CH_4$ assimilation to fewer locations in soil, with increasing areas devoid of methanotrophic activity (Fig. 3.6).

3.4 Discussion

The autoradiographic images presented here are the first high-resolution information available to date on the spatial distribution of methanotrophic activity in upland (aerobic) soils. We demonstrate that the assimilation of atmospheric $^{14}CH_4$ is inhomogenously distributed within the soil profile, both at the smaller (sub-millimeter) and the larger scale (centimeters). The actively $^{14}CH_4$ assimilating bacteria appear to preferentially occupy aggregate surfaces where substrate availability is high, at least in moist soils.

Our results indicate that the activity of $^{14}CH_4$ assimilating microbes is co-regulated by drought and N fertilizer application. The interaction of these factors is complex. Simulated drought extended the zone of $^{14}CH_4$ assimilation into deeper soil layers, while N fertilizers inhibited $^{14}CH_4$ uptake in top soils, but only when soils were dry. This reduction in $^{14}CH_4$ assimilation after N fertilization was accompanied by a restriction of $^{14}CH_4$ uptake to fewer micro-sites, as seen in the frequency distribution of labelling intensity. The experimental treatments drought and N affected soil layers differently, indicating that the regulating factors varied with depth.

Effects of drought

At *Früebüel*, simulated drought led to increased $^{14}CH_4$ assimilation in deeper soil layers relative to control plots. Drought drains pore-channels, resulting in improved gas diffusion from the atmosphere down into the soil profile (Adamsen & King, 1993; Price *et al.*, 2004). Lower soil moisture also reduces the thickness of water films covering soil particle surfaces to which microbes are attached (Young & Ritz, 2000), reducing this important diffusion barrier. Since CH_4 concentrations in the gas phase of

upland soils are already well below atmospheric concentrations, changes in soil moisture have the potential to alter substrate assimilation rates of the presumably severely substrate-limited autotrophic methanotrophic bacteria (Dunfield, 2007). Laboratory experiments have sometimes shown decreases in methanotrophic activity at very low soil moisture (Kammann *et al.*, 2001), but we did not observe such an effect in our field study, despite very severe drought (Hartmann *et al.*, 2010).

Effects of simulated drought differed at *Alp Weissenstein* and *Früebüel*. While CH_4 uptake rates were increased in drought-treated soils, no downward extension of the CH_4 assimilating zone was detected in the autoradiographs. This might be due to the sampling date relative to the fertilizer application. However, we believe that these site-specific differences might also be related to a different drying behaviour of the respective soils (Smith *et al.,* 2000). The soils at *Alp Weissenstein* were lower in bulk density, were less aggregated, and had a higher root density and pore volume than the soils at *Früebüel*. At *Alp Weissenstein,* soils appeared to dry relatively uniformly over the whole profile. In contrast, soils at *Früebüel* dried more quickly in the top layers, whereas the deeper layers stayed moist for longer times. Apart from soil structure, this could be related to several other factors. First, air temperature was higher at the low altitude site *Früebüel,* possibly accelerating evaporation from the soil surface; second, roots were concentrated in the upper part of the profile, so that plant transpiration might have driven the accelerated desiccation of top soils (Doussan *et al.*, 2006; Gerke & Kuchenbuch, 2007). In conclusion, our data suggest that the CH_4-assimilating zone extends further down the soil profile under drought, but that this effect depends on soil properties including soil drainage and desiccation dynamics.

Effects of N fertilizers

Several mechanisms have been proposed by which N fertilizer may affect soil methanotrophic activity (Dunfield, 2007). The most widely endorsed mechanism is inhibition of the enzyme CH_4 mono-oxygenase by ammonia (NH_3; Whittenbury *et al.*, 1970), but there are also indications of other effects, including “salt effects” (Gulledge & Schimel, 1998).

The application of N fertilizers decreased CH_4 assimilation mainly in the top soil, and when soils were dry. This interaction between the applied treatments can potentially be explained by several factors. First, the depth to which the applied fertilizer

infiltrated soils may have been deeper in dry soils compared to moist soils. Dry soils shrink, especially when their clay content is high, and cracks can develop. Moreover, drier soils are often found to be more hydrophobic than moist soils, preventing a rapid absorption of the applied fertilizer solution and allowing deeper penetration. Second, plant growth was reduced in the drought-treated plots (Hartmann *et al.*, 2010), which may have reduced the elimination of NH_4^+ from soil solution by plant uptake. Indeed, both soil NH_4^+ and NO_3^- concentrations were found to be higher under simulated drought than in control plots, in both years of the study (Hartmann *et al.*, 2010). Third, methanotrophic bacteria in the drought-treated plots are likely to have been under more severe osmotic stress. It appears conceivable that methanotrophs may have been more susceptible to effects of NH_3 under these conditions, especially since NH_4^+ concentrations will be higher due to a smaller dilution of the applied fertilizer in less soil solution.

Our main experiment, which factorially combines drought and N fertilizer, has shown little or no effect of N fertilizers on the $^{14}CH_4$ distribution in the shelter-free control plots. Also, there was no clear difference between effects of NH_4NO_3 and urine under simulated drought. However, the fertilizer gradient experiment clearly demonstrated that N effects also occur in moist plots of the same soil, but only at higher N application rates. It further seems that NH_4NO_3 exerts a stronger effect than cattle urine. Effects of NH_4^+-containing fertilizer on the soil methane sink have been reported to depend on soil acidity (e.g. Hütsch, 1996; Hütsch *et al.,* 1993). The application of inorganic NH_4^+-based fertilizers often leads to an acidification of soils (Vitousek *et al.*, 1997). Even in the absence of N application, the soil CH_4 sink has been reported to decrease or to completely vanish when grasslands acidified with time (Hütsch *et al.*, 1994). In our study, the application of NH_4NO_3 led to an acidification especially of top soils, irrespective of the drought treatment (-0.6 and -0.5 pH units within the top 5 cm soil layer at *Früebüel* and *Alp Weissenstein*, respectively) , while urine application did not show any significant changes in soil pH relative to unfertilized plots (Hartmann *et al.*, 2010). It is hard to tell whether soil acidification contributed to inhibitory effects in our main experiment; the absence of inhibitory effects in the drought-treated plots, despite lower pH, suggests that, if present at all, this effect was not so important. However, interactions with soil pH might explain why NH_4NO_3 inhibited the soil CH_4 sink more strongly than cattle urine when applied

in high amounts as in the separate fertilizer gradient at *Früebüel*. A mechanism underlying this effect may have been that soil acidification inhibited nitrification, so that free NH_4^+ was not removed by this process.

$^{14}CH_4$ assimilation patterns and the soil CH_4 sink

The soil CH_4 assimilation rates of the isolated soil cores largely corresponded to the CH_4 fluxes that were measured in the field using *in situ* static chambers (Hartmann *et al.*, 2010). While both the addition of NH_4NO_3 and urine reduced the soil CH_4 sink immediately after application, soil CH_4 uptake completely recovered within weeks, both in control and drought-treated plots. At the time of collection of the soil cores that we ^{14}C-labelled in the laboratory, no effects of N on field-based measurements of CH_4 uptake were detected anymore in plots at *Früebüel*, and effects at Alp Weissenstein were small and independent of the drought treatment (Hartmann *et al.*, 2010). An important conclusion of our study is therefore that the inhibition of CH_4 oxidation in the top soil, which we found when N fertilizers were applied under drought, did not result in corresponding reductions in CH_4 oxidation at the whole soil level. The likely mechanism underlying this apparent discrepancy is that the reduced gas diffusive resistance under drought allowed deeper soil layers to compensate for the lack of CH_4 oxidation in top soils. This compensatory mechanism could not manifest in its full extent under laboratory conditions, because the vertical zone of CH_4 oxidation extended below the labelled core's bottom. Under these conditions, the CH_4 assimilating layers below the soil core could not contribute to CH_4 uptake in the laboratory situation. Thus, a reduction of the CH_4 oxidation rate was detected in the laboratory, but not, or to a lesser extent, in the field. By contrast, when soil moisture is high, deep soil layers cannot compensate for inhibitory effects of NH_4^+ in the top soil. We did not find such inhibitory effects in the drought × fertilizer experiment, probably because plants effectively reduced soil NH_4^+ concentrations when soil moisture was high. However, the higher N application rates in the fertilizer gradient experiment caused a reduction in $^{14}CH_4$ labelling of the soil cores, and this time the laboratory results were consistent with the results from the field (Hartmann *et al.*, 2010).

Some studies have reported delayed effects of N fertilization on soil CH_4 uptake (Gulledge *et al.*, 1997). Assuming that the zone showing inhibited CH_4 uptake

develops by growing from the top of soils, a delayed onset of inhibitory effects can be expected. This is the case because the soil CH_4 sink will only "fail" once methanotrophs in deeper soil layers can not any longer compensate for the loss of methanotrophic activity in the top soil.

3.5 Conclusions

Combining our novel autoradiographic technique with CH_4 flux measurements allowed us to develop a detailed understanding of some of the mechanisms regulating soil CH_4 uptake. While drought increased the soil CH_4 sink by facilitating gas diffusion, complex ecological interactions underlie effects of N fertilizer. First, whether N application resulted in an inhibition of (top soil) $^{14}CH_4$ assimilation depended on drought in our study. Different mechanisms may account for this interaction, but we believe that drought led to reduced plant growth or nitrification rates, a slower removal of the applied N, and therefore higher soil NH_4^+ concentrations. Second, fertilizer effects depended on soil layer, with inhibitory effects being stronger in the top soil. This depth-specific effect is likely to depend on soil hydraulic properties, which control the areas of the soil profile that are infiltrated by the applied fertilizer. Third, and most important, reductions in top soil can be compensated by CH_4 oxidation in deeper layers, but only when soils are dry and diffusive transport to these layers is sufficient. Ultimately, this last point indicates that measurements on bulk soil samples cannot be used to infer on the total soil CH_4 sink.

More generally, any bulk soil quantification of the biomass or activity of methanotrophs, or of other parameters related to their activity, is prone to suffer from the same artifacts if the spatial organisation of soils (e.g. stratification of organisms, processes and mechanisms) is ignored. We speculate that a careful consideration of these issues might help to resolve apparently conflicting published results.

3.6 Acknowledgements

We thank Leo Meile for providing laboratory facilities, Carla Soliva and Samuel Bucher for help with methanogenic cultures, and Patrick Flütsch, Annina Zihler, Carmen Kunz and Annika Ackermann for technical support. Patrick Spielmann,

Daniel Stiehl and Roland Wenger are acknowledged for their help with ^{14}C analysis. Frowin Pirovino and Sarah Wernicke helped to prepare the soil sections. We also are grateful to Ansgar Kahmen and Daniel Stiehl for helpful discussions. This work was founded by Swiss National Science Foundation grant 315230-112681 to PAN. PAN gratefully acknowledges funding by TUMSS (Towards the Understanding of Methane Sinks and Sources), a project of ETH's School Domain of Earth, Environment and Natural Resources. This project was conceived by PAN, the field experiment designed and implemented by AH and PAN, the laboratory techniques developed by PAB with input by PAN and EK, and written up by PAB and PAN with input from the other co-authors.

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3.8 Figures

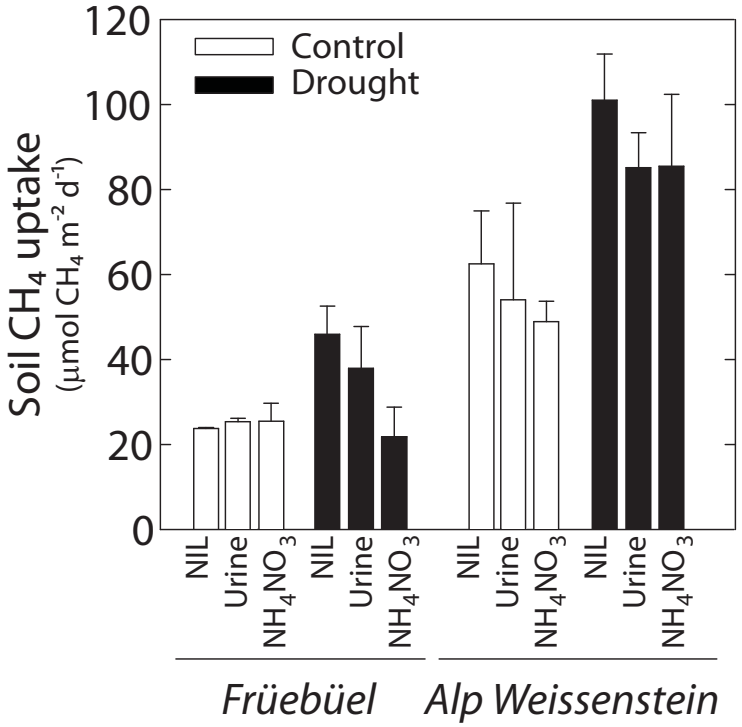

Figure 3.1: Soil CH_4 uptake rates in dependence of site (*Früebüel* or *Alp Weissenstein*), simulated drought (Control or Drought), and N fertilization (NIL, Urine, NH_4NO_3). CH_4 uptake rates were measured on isolated soil cores incubated in the laboratory at 20°C. Error bars show standard errors based on three replicates per treatment combination.

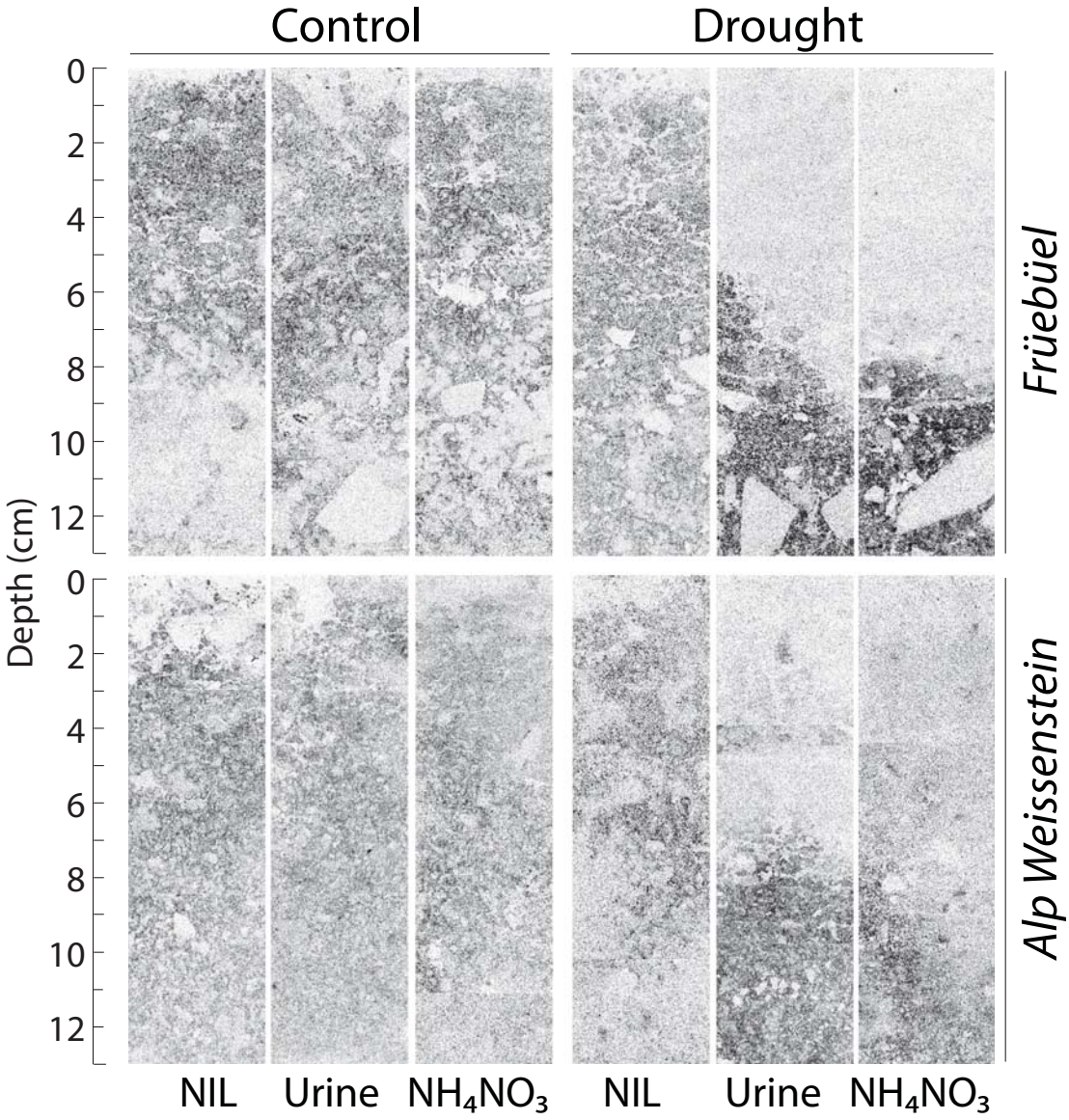

Figure 3.2: Autoradiographs showing the ^{14}C distribution in sections of $^{14}CH_4$-labelled soil cores (h=13 cm), in dependence of site (*Früebüel* or *Alp Weissenstein*), simulated drought (Control or Drought), and N fertilization (NIL, Urine, NH_4NO_3). A representative image out of a total of three autoradiographic images per treatment combination is shown.

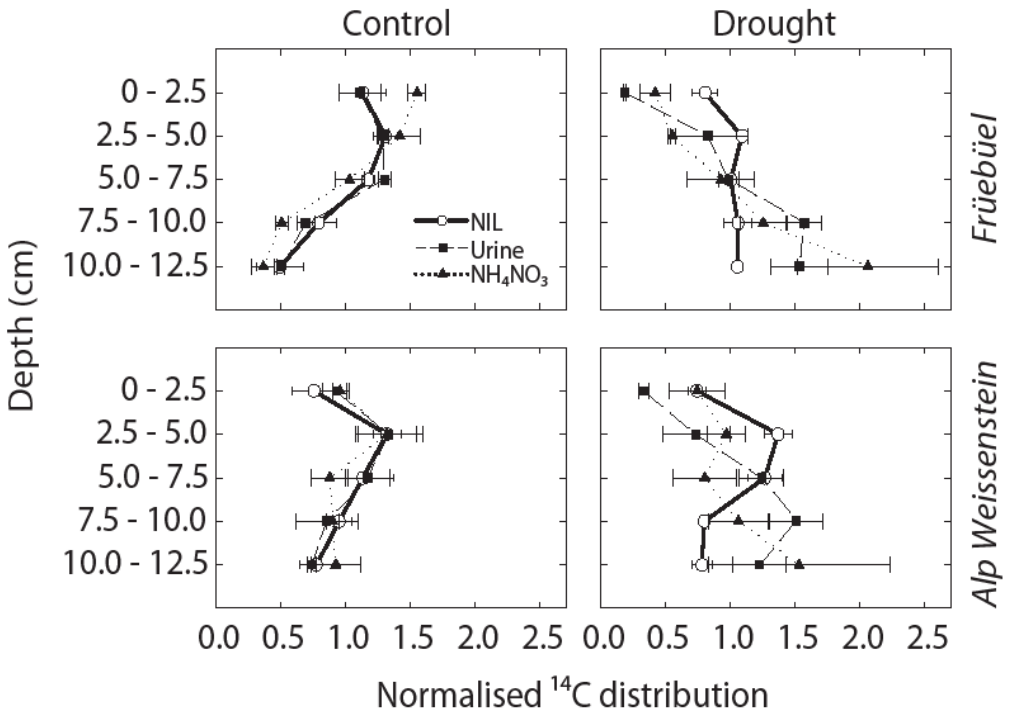

Figure 3.3: Vertical ^{14}C distribution in soil cores labelled with $^{14}CH_4$, in dependence of site (*Früebüel* or *Alp Weissenstein*), simulated drought (Control or Drought), and N fertilization (NIL, Urine, NH_4NO_3). Note that these data are standardised per soil core and thus only show the relative depth distribution (the labelling intensity averaged over all depth layers equals 1.0 for all soil cores). Error bars are standard errors based on three replicates.

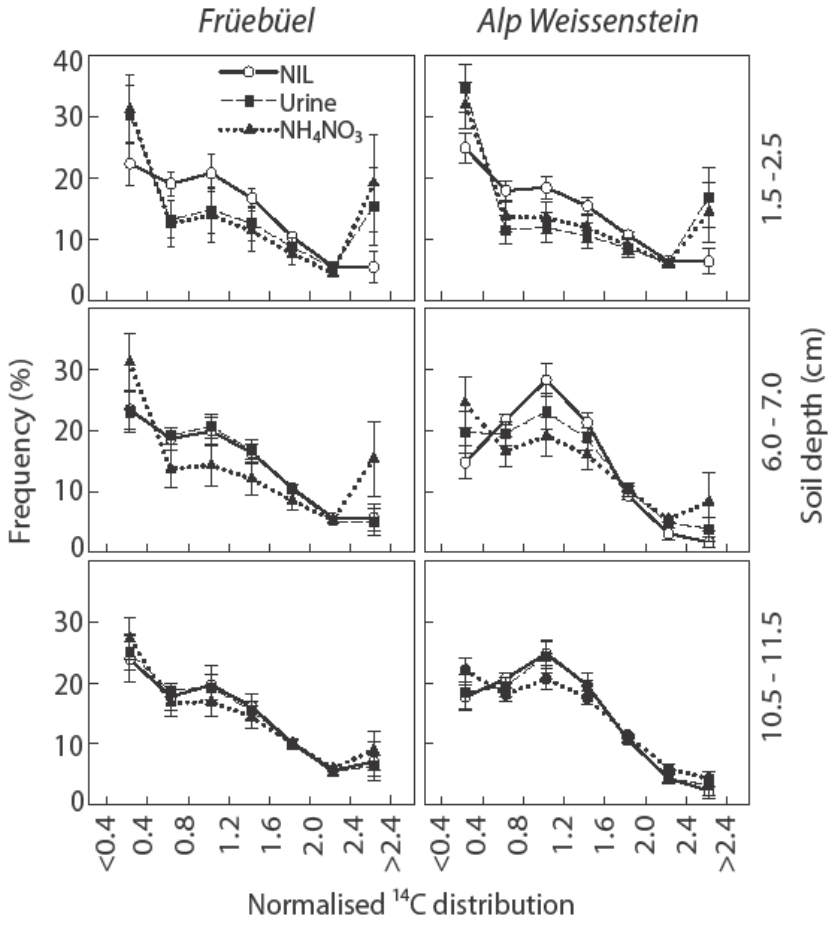

Figure 3.4: Frequency distribution of ^{14}C labelling intensity of 25 µm x 25 µm areas on the autoradiographs of soil sections. Data are shown in dependence of site (*Früebüel* or *Alp Weissenstein*), N fertilization (NIL, Urine, NH_4NO_3) and soil layer. The frequency distribution was calculated by normalising the pixel data for each plot and soil layer, filtering random noise (see Methods for details), and grouping the resulting pixel data into seven labelling intensity classes (<0.4, 0.4-0.8, 0.8-1.2, 1.2-1.6, 1.6-2.0, 2.0-2.4, and >2.4). Error bars are standard errors based on six replicates (data from the drought treatment are included in this figure).

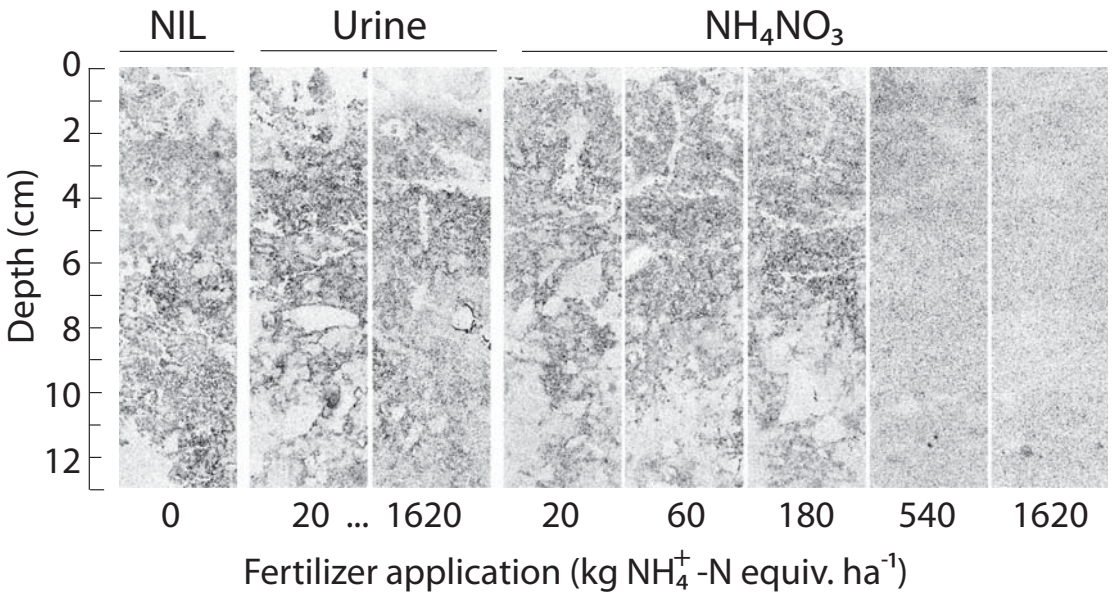

Figure 3.5: Autoradiographic images of soil sections (h=13 cm) from the separate fertilizer gradient experiment at *Früebüel* in which variable amounts of urine and NH_4NO_3 were applied. For the unfertilized plots (NIL), only one out of two plots are shown, since both are virtually equal. For urine, only sections from plots with the lowest and highest N application rate are shown, since increasing amounts of urine did not have any effect on the images obtained. For the NH_4NO_3 treatment, all soil sections are shown.

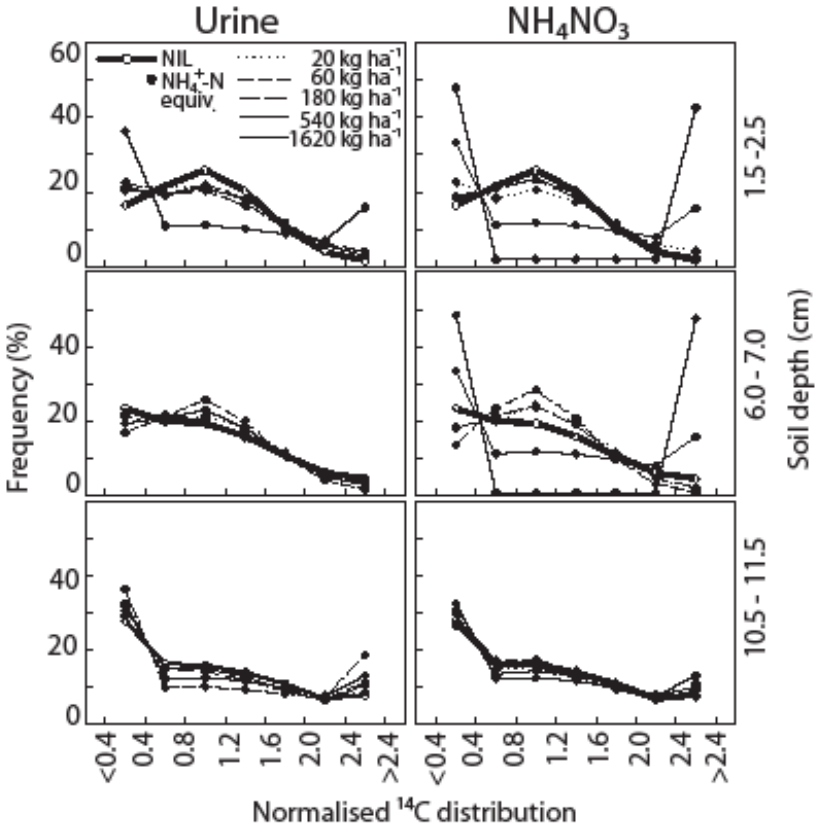

Figure 3.6: Frequency distribution of ^{14}C labelling in autoradiographic images of soil sections from the fertilizer gradient experiment at *Früebüel* in which variable amounts of urine and NH_4NO_3 were applied. See legend of Figure 3.4 for details.

Chapter 4

Effects of N fertilizers and liming on the micro-scale distribution of methane assimilation in the long-term Park Grass experiment at *Rothamsted*

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Chapter 4 was submitted 14 September, 2010.

Abstract

The oxidation of atmospheric methane (CH_4) by soil methanotrophic bacteria constitutes the only biological sink for this greenhouse gas. However, anthropogenic activities, in particular N fertilization, often (but not always) reduce this sink, by mechanisms only partly understood. We argue that the difficulty in developing a process-based understanding of the mechanisms involved is, in part, due to complex interactions with environmental conditions and N cycling, in combination with a lack of information on how the involved processes and organisms are organized within soil structure. We have developed a novel method which permits mapping of the spatial distribution of the active soil methanotrophs at a resolution well below 100µm. In the present study, we applied this technique to a selection of plots from the Park Grass experiment at Rothamsted, UK, to which either no fertilizer, or $(NH_4)_2SO_4$, $NaNO_3$, or manure were applied for over 150 years. We measured the spatial distribution of CH_4 assimilation four times throughout the 2008 growing season, together with field-based measurements of the soil CH_4 sink. In general, methanotrophic activity was most

pronounced within the top 10 cm of soil, and along the surface of aggregates and pore channels. Soil CH_4 oxidation was controlled by soil moisture, with no differences between the plots after correcting for differences in soil moisture within the field site. Exceptions were on the $(NH_4)_2SO_4$-treated plots in which acidification had occurred due to no or little liming. In these plots, methanotrophic activity was restricted to spots in deeper soil layers, which contributed only little to the sink for atmospheric CH_4 due to diffusive limitation of the top soil layers. Our results suggest that spatial distribution of CH_4 assimilation is controlled by local concentrations of NH_4^+ and/or pH within the soil structure. The effect of pH may be direct, or indirect through a reduction in nitrification rates and therefore increased NH_4^+ concentrations, or indirect through a mobilisation of Al^{3+} which also might reduce methanotrophic activity. The concentration of ammonium ions, and their persistence in soil, will depend on the quantity of N applied, its rate of release through mineralization, and its rate of removal by either plant or microbial assimilation or nitrification. Our findings underline the importance of developing a detailed understanding of the spatial organisation of these processes since this will determine the nature and strength of their interactions. The technique we have shown here provides a powerful tool to achieve this goal.

4.1 Introduction

Aerobic soils can act as a sink for atmospheric methane (CH_4) when they harbour soil microbes capable of oxidising CH_4 (Bedard and Knowles, 1989; Hanson and Hanson, 1996). Globally, the soil sink for atmospheric CH_4 is estimated at 30-60 Tg CH_4 yr^{-1} (IPCC, 2001) and is of climatic relevance because, on a per molecule basis, CH_4 contributes 33 times more to greenhouse warming than CO_2 (Shindell *et al.*, 2009).

Soil microbes both produce and consume CH_4 (Megonigal and Guenther, 2008; Mosier *et al.*, 1991). CH_4 consumption generally dominates in well-drained oxic "upland" soils, and these soils therefore act as a net sinks for atmospheric CH_4 (Dunfield, 2007). Although microbes driving soil CH_4 uptake in upland soils have not been isolated and cultured to date, there is a widespread consensus that this sink is essentially driven by soil methanotrophic bacteria, while CH_4 oxidation by other soil microbes is probably only of minor importance (Bédard and Knowles, 1989; Bender and Conrad, 1992).

In most soils, the diffusion of atmospheric CH_4 to the sites occupied by methanotrophs limits CH_4 uptake. In the absence of methanogenesis in the subsoil, the activity and distribution of these microbes in the soil profile is therefore limited by access to atmospheric CH_4 (Striegl, 1993). Soil texture (Doerr *et al.*, 1993), aggregation and porosity (Ball *et al.*, 1997; Boeckx *et al.*, 1997) therefore often explain differences in soil CH_4 uptake between sites. The solubility of CH_4 in water is low and diffusion of CH_4 in soil solution orders of magnitude slower than in the gas phase. Therefore, soil CH_4 uptake is limited by diffusion when moisture is high (Young and Ritz, 2000). Accordingly, soil moisture is generally the single most important factor explaining temporal variability in soil CH_4 uptake. Very low soil moisture, on the other hand, can limit the activity of soil methanotrophs by physiological stress, so that the soil CH_4 sink can also decline under these conditions, despite high soil gas diffusive conductance (Kammann *et al.*, 2001; Schnell and King, 1996).

Anthropogenic activities have reduced the soil CH_4 sink in many ecosystems, primarily through the application of mineral nitrogen fertilizers and through physical disturbance of agricultural soils (Hütsch *et al.*, 1994). The main mechanism that has

been proposed to explain the detrimental effect of NH_4^+ on soil CH_4 uptake is competitive binding of NH_3 to methane mono-oxygenase, a key enzyme in the assimilation pathway of methanotrophic bacteria (O'Neill and Wilkinson, 1977; Whittenbury *et al.*, 1970). However, this mechanism cannot explain observations from many studies where the addition of N fertilizer did not inhibit, or even increased, soil CH_4 uptake (Bodelier and Laanbroek, 2004; Gulledge and Schimel, 1998). For example, the applications of organic manures leading to the release of large amounts of NH_4^+ had no (Hütsch *et al.*, 1994; Hütsch *et al.,* 1996) or even a positive effect (Willison *et al.*, 1996) on the soil CH_4 sink in long-term agricultural trials.

Soil acidity plays an important role in controlling soil CH_4 oxidation, but the mechanisms involved remain unclear. It is possible that high acidity affects soil methanotrophs directly, but soil acidity also interacts with processes of the soil N cycle and with effects of N application. Some acidic soils do not show atmospheric CH_4 uptake at all (Powlson *et al.*, 1997), whereas many soils with pH around 4 show very high oxidation rates (e.g. alpine pasture: Braun et al., 2010; Dobbie *et al.,* 1996). In the long term, the application of NH_4^+-based fertilizers leads to soil acidification. In studies where this happens, it often remains unclear whether decreases in CH_4 uptake result from increased NH_4^+ concentrations, low soil pH, or a combination of both.

In conclusion, effects of NH_4^+ on methanotrophic bacteria have been studied in great detail. While enzymatic effects of NH_4^+ are comparatively well understood in simple laboratory settings, the mechanisms controlling CH_4 oxidation under field conditions are less clear. In part, this is because the different processes involved seem to interact in a complex way, potentially leading to apparently ambiguous findings.

A crucial piece of information missing from most studies, and therefore complicating the interpretation of results, is how the investigated processes and micro-organisms are organised in space and time. Whether reductions in the soil CH_4 sink can be expected following NH_4^+ application will, for example, depend on whether the fertilizer applied reaches the locations occupied by the active methanotrophic bacteria. In turn, this will depend on soil permeability and fertilizer infiltration characteristics, the dynamics of removal of NH_4^+ by plant and microbial assimilation and nitrification, and the local environmental conditions including soil moisture and acidity. Soils are extremely heterogeneous, and conditions vary both at the large scale

within the soil profile as well as at the micro-scale around aggregates, roots, pore channels, and plant residues. It therefore appears likely that the ecological mechanisms controlling the activity of soil methanotrophs also vary at the same spatial scales.

To date, the spatial distribution of soil methanotrophic activity has largely been studied at the level of soil horizons. For example, Roslev et al. (1997) labelled soils with $^{14}CH_4$ and measured the assimilated activity in different soil layers. In several other studies, methanotrophic activities were quantified as CH_4 oxidation rates of sieved soil layers (Adamsen and King, 1993; Bender and Conrad, 1994; Whalen *et al.*, 1992). However, this method will potentially lead to inaccurate estimates of *in situ* activity because the gas diffusive resistance originating from the soil layers above is missing and the environmental conditions altered (Bradford *et al.*, 2001; Roslev *et al.*, 1997).

We have developed a method to allow mapping of the spatial distribution of methanotrophic activity in intact soil cores, at a resolution below 100 µm (Braun *et al.*, 2010). In combination with field-based measurements of CH_4 fluxes and processes of the N cycle, this facilitates a detailed understanding of N application-effects in pastures (e.g. Braun *et al.,* 2010, Hartmann *et al.*, 2010a,b).

In the present study, we applied this novel approach to soils from the Park Grass experiment at Rothamsted, UK. In parallel, soil-atmosphere CH_4 fluxes were measured using static chambers. Park Grass is the longest running ecological experiment in the world (Silvertown *et al*., 2006) and is ideally suited to studying effects of management on soil methanotrophs, since these bacteria have extremely low growth rates and decades to centuries are needed until their communities have adapted to the new conditions (Menyailo *et al.*, 2008; Mosier *et al.*, 1997). The Park Grass experiment consists of plots with various combinations of fertilizer and liming treatments; our objectives were to test for effects of different forms of N application on CH_4 oxidation rates and on the spatial distribution of soil methanotrophs, and to test whether these depended on the acidity of the investigated soils.

4.2 Material and Methods

Field site and experimental design

Park Grass experiment at Rothamsted Research was established in 1856, after the site had been in permanent grassland for at least a century. The top soil (0-23 cm) is a silty clay loam containing 22% clay, 29% silt and 49% sand (Tye *et al.*, 2009) and has an organic C content of 2.7-6.6%, depending on the long-term treatments on the different plots. It is moderately well drained and is classified as an Aquic Paleudalf in the USDA classification. Mean annual air temperatures averaged ca. 9.1°C in 1878-1990 and have increased to ca. 10.5°C in 2001-5 (5-year averages); annual precipitations averaged ca. 700 mm from 1971-2000 (data published online at UK Environmental Change Network http://www.ecn.ac.uk and Rothamsted Research http://www.rothamsted.ac.uk).

The experiment is divided into plots treated with different fertilizer combinations. The present study used an unfertilized control plot ("NIL" treatment, plot 3) and three plots treated with either $(NH_4)_2SO_4$ (plot 1), $NaNO_3$ (plot 17), or manures (plot 13/2). The inorganic fertilizers are applied once per year in spring as a single application containing 48 kg N ha^{-1}. In 2008 they were applied on April 3. The organic manure is applied biannually, alternating between farmyard manure (containing ca. 240 kg N ha^{-1}, last application in February 2005) and poultry manure (65 kg N ha^{-1}, last application in February 2007, one year before this study). Since 1965, each of these plots has been subdivided into four subplots, which are adjusted to a top soil (0-23cm) pH of approximately 7, 6 or 5 by differential liming where necessary (subplots a, b and c), or left unlimed (subplot d). Lime was last applied in November 2005. As is typical for very long-term experiments, the management was occasionally altered based on the results obtained, and to address new scientific questions. Full details can be found in the Guide to the Classical and other Long-term Experiments, Datasets and Sample Archive (http://www.rothamsted.ac.uk and http://www.era.rothamsted.ac.uk/parkgrass_1.html). We measured soil CH_4 fluxes and the spatial distribution of the active soil methanotrophs four times during the growing season of 2008. Soil sampling and flux measurements were generally conducted on consecutive days, except for the last soil sampling which was delayed by heavy rain.

CH_4 flux measurements

Soil CH_4 uptake was measured *in situ* during four periods between March and June 2008. The static chambers used consisted of cylindrical PVC collars (31.8 cm diameter × 25 cm height) that could be closed with lids. To install the collars, the soil was cut with a narrow spade and the collars pushed 15 cm into the ground. To measure CH_4 flux rates, the chambers were closed and headspace samples collected after 5, 20 and 35 minutes and analysed by gas chromatography (Agilent 6890N equipped with an FID detector, Agilent Wilmington Delaware, U.S.A.). CH_4 uptake rates were calculated by linear regression of CH_4 concentration vs. time.

The chamber collars were removed after the first series of measurements to allow the mechanised application of N fertilizers. After that, the collars were re-installed at a new location within the same plots where they remained for the following three sampling periods.

Soil sampling

On March 27, April 11, May 11 and September 12, 2008, one soil core was sampled in each plot by carefully inserting polyethylene tubes (16 cm length x 5.7 cm internal diameter) into pre-cut soil. The sampling tubes were then carefully excavated with a spade to minimise soil disturbance. The soil cores were capped immediately at both ends and transferred to the laboratory for further analysis.

Soil moisture, temperature and acidity

Concomitantly with gas fluxes measurements and soil sampling, top soil moisture and temperature were measured (Theta Probe HH2, Delta-T Devices, Cambridge, UK and GTH 175 digital thermometer with Pt-1000 probe, Greisinger electronic, Germany).
On March 11-12, 2008, 15 cores (2 cm diameter × 23 cm length) were collected per subplot; these were bulked, air-dried, milled to a particle size < 2 mm and pH measured in water (10 g air-dry soil in 25 mL H_2O).

$^{14}CH_4$ labelling

After equilibrating to laboratory conditions (20°C), the collected soil cores were each labelled with a total activity of ca. 100 kBq $^{14}CH_4$, which required 7 days. The labelling was conducted in gas-tight jars (3L), in which the headspace concentration was kept below 8 μL CH_4 L^{-1}. O_2 concentrations were maintained between 15-20% to

ensure aerobic conditions. CO_2 from microbial respiration was trapped in a plastic tube containing 1.5 M NaOH placed within the jar. For further details, see Braun et al. (2010).

The $^{14}CH_4$ applied was produced by cultures of the methanogenic archaea *Methanobacterium thermoautotrophicum* (DSM 1053; Zeikus and Wolfe, 1972) fed with $NaH^{14}CO_3$ (specific activity of 1.48-2.22 GBq $mmol^{-1}$, Perkin Elmer, Schwerzenbach, Switzerland), following the protocol of Daniels and Zeikus (1983). The potential by-products ^{14}CO and $^{14}CO_2$ were removed with Hopcalite™ (Dräger, Germany; Harder, 1997) and sodium hydroxide (NaOH) solution, respectively.

Soil sections

After labelling, the 15 cm soil cores were freeze-dried and cast in epoxy resin (Laromin C 260, BASF, Ludwigshafen, Germany, mixed at a ratio of 2:3 with Araldite DY 026SP hardener, Astorit AG, Einsiedeln, Switzerland). To improve the impregnation of the soil cores, these were evacuated to a pressure of 25 kPa and slowly brought back to atmospheric pressure. The resin was left hardening for at least 4 days. The resin core (still in its sampling tube) was cut horizontally into three sections of 5 cm height (Discoplan diamond circular saw, Struers GmbH, Birmensdorf, Switzerland). A vertical slice was cut from the centre of each of these sections, resulting in three sections of 5 cm x 5 cm surface area and 1 cm thickness. Each section was mounted on a glass slide and the surface levelled with a diamond cup mill until the maximum deviation from planarity was below 30 μm. After that, the surface of these sections was further polished with silicon abrasive paper.

Autoradiographs of the embedded soil sections were obtained by exposing phosphor imaging plates (BAS III S, Fuji Film AG, Dielsdorf, Switzerland) for 10 days and digitising these at a resolution of 50 μm with a red-excited fluorescence scanner (Storm 840, Molecular Dynamics GmbH, Krefeld, Germany). The resulting scans were corrected for background exposure and random noise reduced with a smoothing function; we refer to Braun et al. (2010) for details of these procedures.

The autoradiographs were compared with conventional digital photographs, allowing the identification of soil features including soil aggregates, soil pores, plant roots and stones. A few soil cores showed cracks that may have been the result of soil sampling

- the area around these cracks was excluded from data analysis to avoid potential artifacts.

First, we analysed the vertical distribution of assimilated ^{14}C (i.e. labelling as a function of soil depth) in a 3 cm wide vertical strip, thus excluding a border of ca. 1 cm on each side of the section to avoid potential edge-effects (although none were obvious from visual inspection). If there were features (e.g. stones) larger than 0.5 cm, these were also excluded from the analysed area. The vertical profile of ^{14}C assimilation was then normalized to an average of 1.0. This normalisation procedure was done because the amount of label effectively taken up by the methanotrophs varied slightly between samples.

Second, the small-scale (horizontal) ^{14}C distribution was compared between treatments. This was achieved by analysing the frequency distributions of the labelling intensity in three 3 cm wide areas per soil core (located at 1.5-2.5, 6-7 and 10.5-11.5 cm depth). The background-corrected data was again normalised to an average of 1.0 per analysed area and histograms generated by aggregating the labelling intensity into seven classes (<0.4, 0.4-0.8, 0.8-1.2, 1.2-1.6, 1.6-2.0, 2.0-2.4, and >2.4). More details regarding this procedure can be found in Braun et al. (2010).

Soil sections

After labelling, the 15 cm soil cores were freeze-dried and cast in epoxy resin (Laromin C 260, BASF, Ludwigshafen, Germany, mixed at a ratio of 2:3 with Araldite DY 026SP hardener, Astorit AG, Einsiedeln, Switzerland). To improve the impregnation of the soil cores, these were evacuated to a pressure of 25 kPa and slowly brought back to atmospheric pressure. The resin was left hardening for at least 4 days. The resin core (still in its sampling tube) was cut horizontally into three sections of 5 cm height (Discoplan diamond circular saw, Struers GmbH, Birmensdorf, Switzerland). A vertical slide was cut from the centre of each of these sections, resulting in three sections of 5 cm x 5 cm surface area and 1 cm thickness. Each section was mounted on a glass slide and the surface levelled with a diamond cup mill until the maximum deviation from planarity was below 30 μm. After that, the surface of these sections was further polished with silicon abrasive paper.

Autoradiographs of the embedded soil sections were obtained by exposing phosphor imaging plates (BAS III S, Fuji) for 10 days and scanning these at a resolution of 50 μm (Storm 840, Molecular Dynamics GmbH, Krefeld, Germany). The resulting scans were corrected for background exposure and random noise reduced with a smoothing function; we refer to Braun et al. (2010a and 2010b) for details of these procedures.
The autoradiographs were compared with conventional digital photographs, allowing the identification of soil features including soil aggregates, soil pores, plant roots and stones. A few soil cores showed cracks that may have been the result of soil sampling - the area around these cracks was excluded from data analysis to avoid potential artifacts.

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Statistical analysis

From a statistical point of view, the Park Grass experiment suffers from a lack of replication for most of the fertilizer combinations (Powlson, 1994). However, it is unique in that no other ecological experiment is available with such a well-documented long-term management history. Crawley et al. (2005) argue that the design shortcomings are partly alleviated by the large size and relative uniformity of the plots.

Our analysis considers the subplots of different soil pH as independently randomised (which they are not). For the gas flux data, the four repeated measurements in time were treated as replicates. For the analysis of the depth distribution of ^{14}C assimilated, the residuals of the data for the depth intervals were treated as independent. For all models fitted, we strived not to inflate the degrees of freedom in the analysed data set more than was absolutely necessary. All effects we discuss here were statistically highly significant ($P<0.001$ in most cases); since, however, these tests are biased, we prefer to discuss most results without giving the actual P values. While our results therefore have to be treated with caution, fortunately the effects detected were very obvious, so that we believe that the risk of false inferences is negligible.

All data were analysed with maximum likelihood models as implemented in the *lme* procedure of the *nlme* package of R 2.10.1 (R Development Core Team 2010). Subplot, subplot × time, or subplot × depth (i.e. soil layers) were treated as random effects (see above), while fertilizer, the liming treatment, and, where appropriate, depth and histogram class, were fixed effects. In several analyses, we also fitted the actual top soil pH or soil moisture as covariates – this is indicated with the respective results.

4.3 Results

Soil CH_4 uptake

Soil CH_4 uptake ranged from 0 to approximately 60 µmol CH_4 m^{-2} d^{-1}, depending on sampling date and treatment (Fig. 1). Soil moisture was high during all except the sampling in early May, and accordingly soil CH_4 oxidation rates were low on these occasions.

The effect of the liming treatments on CH_4 oxidation (subplots a to d) depended on the fertilizer applied ($P<0.001$ for fertilizer × pH). Under $(NH_4)_2SO_4$ application, soil pH was very low when no lime was applied (subplot d; Table 1) and soil CH_4 uptake rates were reduced in subplots c and d where pH was lowest (apart from 3c). In the $NaNO_3$-fertilized plot, an apparently reverse effect was found (Fig. 1), but this reversal was essentially driven by differences in soil moisture that developed due to a

slight depression in terrain towards subplots a and b and consequently higher soil moisture.

This correlation becomes more obvious when the measured CH_4 uptake rates are plotted against soil moisture at the time of the flux measurements (Fig. 2). In general, soil moisture was a very good predictor of soil CH_4 uptake, regardless of the applied fertilizer, except for subplots c and d of the $(NH_4)_2SO_4$ treatment (the plots with lowest soil pH) in which CH_4 uptake was substantially lower than expected at times when rates in other plots were generally high. The variance explained by this regression increases from 70% to 90% when these two subplots are excluded, reflecting the additional inhibition of CH_4 uptake in these subplots.

Soil temperature was 6.9±0.1°C, 8.6±0.1 °C, 16.4±0.1°C and 14.9±0.1 °C on the four sampling periods, and had no significant effect on the measured CH_4 fluxes.

^{14}C distribution in soil cores

Our ability to adequately label the collected soil cores depended on a sufficiently high CH_4 assimilation rate of the soil. For the unlimed subplot d of the $(NH_4)_2SO_4$ treatment, these conditions were only met during the 3rd sampling where soils were comparably dry. No data are therefore available for this subplot on the other dates.

The depth of the collected cores included virtually the entire zone which assimilated atmospheric CH_4 (Fig. 3). The assimilation of $^{14}CH_4$ was heterogeneous, with a tendency for increased activity on the surface of aggregates and along small pore channels. However, the centre of soil aggregates was also labelled. Soil features such as roots or small stones did not appear to alter the distribution of ^{14}C.

The vertical distribution of ^{14}C showed a distinct profile and varied between dates. When soil moisture was lowest (May 11, 2008), the maximum labelling was found at a depth of 5-10 cm. Under higher soil moisture, more ^{14}C was recovered closer to the soil surface. In general, soil pH and fertilizer type did not alter the vertical distribution of assimilated $^{14}CH_4$. Exceptions were the $(NH_4)_2SO_4$-fertilized subplot c in which $^{14}CH_4$ assimilation was also found in deeper soil layers. In fact the $(NH_4)_2SO_4$ - fertilized subplot d (with a soil pH of 4.0) showed only significant $^{14}CH_4$ assimilation below 9 cm depth (Fig. 4).

The analysis of the small-scale distribution of assimilated ^{14}C revealed significant effects of depth ($P<0.001$, Fig. 5). In general, the assimilation of ^{14}C was restricted to fewer sites in deeper soil layers. No effects of fertilizer and liming treatment were detected with the exception of a restriction of ^{14}C assimilation to fewer spots in the acidic subplots of the $(NH_4)_2SO_4$ treatment: the restriction was only observed in the third and fourth samplings (May 11 and September 12, 2008).

4.4 Discussion

Our field measurements of soil-atmosphere CH_4 exchange indicate that the investigated soils from the Rothamsted Park Grass experiment are net sinks for atmospheric CH_4, at least when soil moisture is low. The autoradiographic technique we have developed shows that the active methanotrophs are heterogeneously distributed in soils, with CH_4 assimilation being concentrated in the top 10 cm of the profile. Zones of increased CH_4 assimilation were found on the surface of aggregates and along small pore channels, probably reflecting the easier access to atmospheric CH_4 due to lower gas diffusive resistance at these sites. $(NH_4)_2SO_4$ fertilizer decreased CH_4 assimilation in plots with pH≤5 to a smaller area. With lower pH, the inhibitory effect of $(NH_4)_2SO_4$ increased.

Methanotrophic bacteria showing the "high affinity" uptake kinetics observed in upland soils (Bender and Conrad, 1993) have not yet been successfully cultured under laboratory conditions (Knief and Dunfield, 2005), probably due to their very low growth rate. One might speculate that such an oligotrophic life style necessitates a spatial niche where these bacteria are protected from biomass loss due to grazing, and that the inside of soil aggregates might be such a place. However, this is not supported by our autoradiographic images.

The data presented here are the first field-based CH_4 flux measurements in Park Grass. The results differ somewhat from those of Hütsch et al. (1994) who measured CH_4 uptake in laboratory incubations, using both soil cores and sieved soil, from a series of Park Grass plots which received the same N fertilizers as in our study, but at higher rates (96-144 kg N ha^{-1} yr^{-1}) and were combined with a full supply of other nutrients (P, K, Mg). In contrast to our results, the inhibition of CH_4 uptake was complete in all

NH_4^+ - fertilized plots studied by Hütsch et al. Several factors may account for this. First, the N application rates were higher (≥96 kg N ha^{-1} yr^{-1} compared to 48 kg N ha^{-1} yr^{-1} in the plots we used). A second possibility is that the remaining zones of methanotrophic activity were below the soil depth sampled by Hütsch et al. (1994) in their laboratory incubations (10 cm). This possibility seems likely, especially since our autoradiographic analyses show that methanotrophy becomes restricted to deeper soil layers under NH_4^+ fertilization and acidification.

Soil moisture was the single most important factor controlling soil CH_4 uptake, except when soils were strongly acidified by the addition of $(NH_4)_2SO_4$ and where no lime was applied to correct this. CH_4 diffusion in soils occurs mainly in the gas phase, since the solubility of CH_4 in water is low and diffusion in the liquid phase comparably slow. Combined with the fact that atmospheric CH_4 is only available to soil methanotrophs at sub-atmospheric concentrations and so limits their activity (Dunfield, 2007; Torn and Harte, 1996), it is not surprising that soil moisture was found to be the dominant control of soil CH_4 uptake (Bradford *et al.*, 2001; Regina *et al.*, 2007). The soils of Park Grass were never really dry during our sampling, so we did not encounter conditions under which methanotrophic activity could have ceased due to a very low water potential (e.g. Kammann *et al.,* 2001). However, other experiments that we have conducted have shown that a reduction of ecosystem-level CH_4 uptake is rarely found even under very dry conditions, because deeper, less desiccated soil layers can compensate for reductions in CH_4 oxidation in the top soil (Braun *et al.*, 2010; Hartmann *et al*., 2010).

Several mechanisms may explain the absence of methanotrophs in the top soil of the acidified subplots of the $(NH_4)_2SO_4$ treatment. A number of studies have reported negative effects of acidification on the soil CH_4 sink (Benstead and King, 2001, Bradford *et al.* 2001). In the Park Grass experiment, Hütsch et al. (1994) found a negative effect of soil acidification in subplot 7 d which receives all fertilizers except N and remains unlimed (but legumes proliferated under these conditions, introducing N to the ecosystem by symbiotic dinitrogen fixation). This subplot, and also subplot c with a pH of 5.0 showed a complete inhibition of CH_4 uptake. If NH_4^+ is the key compound that controls methanotrophic activity, changes in the activity of nitrifying micro-organisms will be of particular importance since these can prevent high soil

NH_4^+ concentrations. The optimum pH for autotrophic ammonia oxidising bacteria is in the neutral to slightly alkaline range (Nicol *et al.,* 2008), and their activity is generally inhibited at low pH (Kowalchuk and Stephen, 2001) as found in the unlimed Park Grass plots (Johnston *et al.* 1986). By contrast, ammonia oxidising archaea have recently been shown to dominate the nitrification process in many soils (Leininger *et al.*, 2006; Prosser *et al.,* 2008), especially under acidic conditions (pH 4.9; Nicol *et al.,* 2008). In unfertilized Park Grass soil from plots 7c and d (pH 5.0 and 4.8, respectively), soil incubation experiments showed increased NH_4^+ accumulation because nitrification was inhibited (Hütsch *et al.,* 1994). It is, however, not clear whether NH_4^+ also accumulated in the field where lower temperatures will limit N mineralization and thus production of NH_4^+. The concentrations of NH_4^+ in soil extracted at the start of the incubation suggest otherwise (Hütsch *et al.,* 1994). However, these data were collected at the end of March, when plants were vigorously growing, and there are likely to be periods in the year in which plant uptake does not effectively remove free NH_4^+ and concentrations harmful to methanotrophs might build up. Another possible explanation for the reduced soil CH_4 sink in the acidic plots of the Park Grass experiment receiving $(NH_4)_2SO_4$ is an increased mobilization of Al^{3+} under acidic conditions (Blake *et al.*, 1994) which might inhibit soil CH_4 oxidation (Tamai *et al.*, 2007). In the unfertilized, unlimed subplot 3d (which is part of the present study), soil extractable Al^{3+} as well as the Al-content of harvested hay massively increased when soil pH declined to below 4.5-5.0 in $CaCl_2$ in the late 1970's (Blake *et al.,* 1994).

How N fertilizers affect soil CH_4 uptake under field conditions is still subject to debate. While NH_3 can competitively bind to the enzyme responsible for CH_4 assimilation in methanotrophs and thus reduce CH_4 oxidation, the mechanisms are clearly more complex in the field. In their comprehensive review, Bodelier and Laanbroek (2004) compiled studies of effects of NH_4^+ application and show that, in some cases, effects occurred immediately upon application but in others after a delay; effects could be either transient or permanent, and finally there were studies showing no effect or even a positive response to NH_4^+ fertilization. In our study, we were only able to radio-label soil from the unlimed $(NH_4)_2SO_4$ plot when the subsoil was not water-saturated. Since the autoradiographs were produced from soil labelled almost a full year after the last fertilizer application (1st sampling), the inhibition of

methanotrophy in the top soil appears to be permanent in this plot – certainly not a transient effect of the added NH_4^+. Conversely, the differences in spatial distribution of methanotrophic activity in the fertilizer treatments in our plots suggest that the ecosystem-level CH_4 sink will respond differently to changes in soil moisture. Upon wetting, soil CH_4 uptake may decrease more rapidly when the active methanotrophs are absent in the top-soil. However, these effects will strongly depend on the drying-re-wetting dynamics and the hydraulic properties of the respective soils.

In contrast to the strongly acidified subplot d of the $(NH_4)_2SO_4$ treatment of Park Grass, where the inhibition of CH_4 oxidation in the top soil appears to be permanent, in a field study in alpine pasture soils in Switzerland we detected a transient inhibition of $^{14}CH_4$ uptake in the top soil, which manifested only for a few days to weeks in measured ecosystem level fluxes. Top soil acidification upon NH_4NO_3-application in that study had reached levels exceeding the conditions in Park Grass subplots c (Braun *et al.,* 2010; Hartmann et al. 2010). A similar transient effect also appears possible in Park Grass, but we believe that its occurrence will depend on how the fertilizer is applied (e.g. as aqueous solution or in solid form) and on soil infiltration characteristics. These factors will determine the temporal and spatial distribution of NH_4^+ in soil solution.

The application of farmyard manure also releases substantial amounts of NH_4^+, but this does not appear to result in an inhibition of the soil CH_4 sink (this study, Hütsch *et al.*, 1993; Willison *et al.*, 1996). NH_4^+ is slowly released from manure so that the soil solution NH_4^+ concentrations will be lower than after inorganic fertilizer application. Manure application also does not lead to soil acidification, or does so only slowly, so that soil NH_4^+ concentrations might be kept low by high nitrification rates. The immobilisation of NH_4^+ in microbial biomass might also be stimulated by the increased organic substrate supply from manure (Willison *et al.*, 1996). In addition, manure will increase the total microbial population compared to soil only receiving inorganic fertilizers. So, even if CH_4 oxidizers are a small fraction of the total population, total activity will be greater than in a treatment without manure. In this sense, a manure-treated soil could be considered to be more resilient to the factors tending to decrease CH_4 oxidizing activity.

Based on our data, we can not unequivocally attribute the inhibited CH_4 assimilation (in the acidified $(NH_4)_2SO_4$-fertilised subplots) to a direct effect of NH_4^+ or to acidification. The soil acidification front develops from the soil surface, and soil NH_4^+ concentrations also are highest there, especially after fertilizer application (Blake *et al.,* 1999). Regardless of the exact mechanism involved (competitive inhibition by NH_4^+, toxic effects of Al^{3+}, low soil pH, or related processes), we expect methanotrophs to be inhibited in the top soil first. The autoradiographs from the acidified subplots (Fig. 3) suggest that this indeed is the case. In any soil, the niches in deeper soil layers occupied by methanotrophs will be controlled by diffusion limitation as atmospheric CH_4 has to enter the soil from the surface. Soil moisture in the subsoil of the Park Grass Experiment is high for most of the time, thus limiting the vertical niche suitable for atmospheric CH_4 oxidation. While one could expect methanogenesis under these conditions, we did not detect net CH_4 emissions during the course of our measurements.

4.5 Conclusions

This is the first study to analyse the small-scale distribution of methanotrophic activity under fertilisation and liming. Our results demonstrate that the long-term application of NH_4^+-based fertilizers (unlike NO_3^--based or organic fertilizers) alters the spatial distribution of CH_4 assimilation and the total CH_4 sink. Our data, in combination with other published results from the same site, suggests that the spatial distribution of CH_4 assimilation is controlled by local concentrations of NH_4^+ and/or pH within the soil structure. Concentrations of ammonium ions, and their persistence in soil, will depend on the quantity of N applied, its rate of release, and its rate of removal by either plant or microbial assimilation or nitrification. These factors will interact with soil structure and moisture content which control movement of CH_4 to potential oxidation sites as well as the various N cycle processes. These findings underline the importance of developing a detailed understanding of the spatial organisation of these processes since this will determine the nature and strength of their interactions. The technique we have shown here provides a powerful tool to achieve this goal.

4.6 Acknowledgements

Keith Goulding and the Lawes Trust are gratefully acknowledged for their hospitality at Rothamsted and for allowing us to work in the Park Grass experiment. Rothamsted Research is a grant-aided institute of the UK Biotechnology and Biological Sciences Research Council. Leo Meile provided laboratory facilities and Carla Soliva and Samuel Bucher helped with methanogenic cultures. We further thank Patrick Flütsch, Annina Zihler, Carmen Kunz and Annika Ackermann for technical support. Patrick Spielmann, Daniel Stiehl and Roland Wenger kindly allowed us to use their equipment for ^{14}C analyses. Frowin Pirovino provided infrastructure for the mechanical preparation of the soil sections. This work was funded by a Swiss National Science Foundation grant 315230-112681 to PAN and the Walter Hochstrasser Foundation. PAN gratefully acknowledges funding by “TUMSS” (Towards the Understanding of Methane Sinks and Sources), a project of ETH's School Domain of Earth, Environment and Natural Resources. This project was conceived by PAN. The laboratory techniques were developed by PAB with input by PAN. This project was written up by PAB and PAN with input from DSP and PRP.

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4.8 Tables and Figures

Table 4.1: Soil pH (in water) in air dried soil of the four investigated plots at Park Grass. The plots were sampled on March 11-12, 2008.

Plot	N treatment	Liming treatment (target pH)			
		subplot a	subplot b	subplot c	subplot d
		7.0	6.0	5.0	no liming
3	NIL (Control)	7.2	6.1	4.9	5.2
1	$(NH_4)_2SO_4$	7.1	6.1	5.0	4.0
17	$NaNO_3$	7.0	6.2*	5.7**	5.9
13/2	Manure	6.9	5.9	5.1	5.2

*No lime applied since 1964

**No lime applied

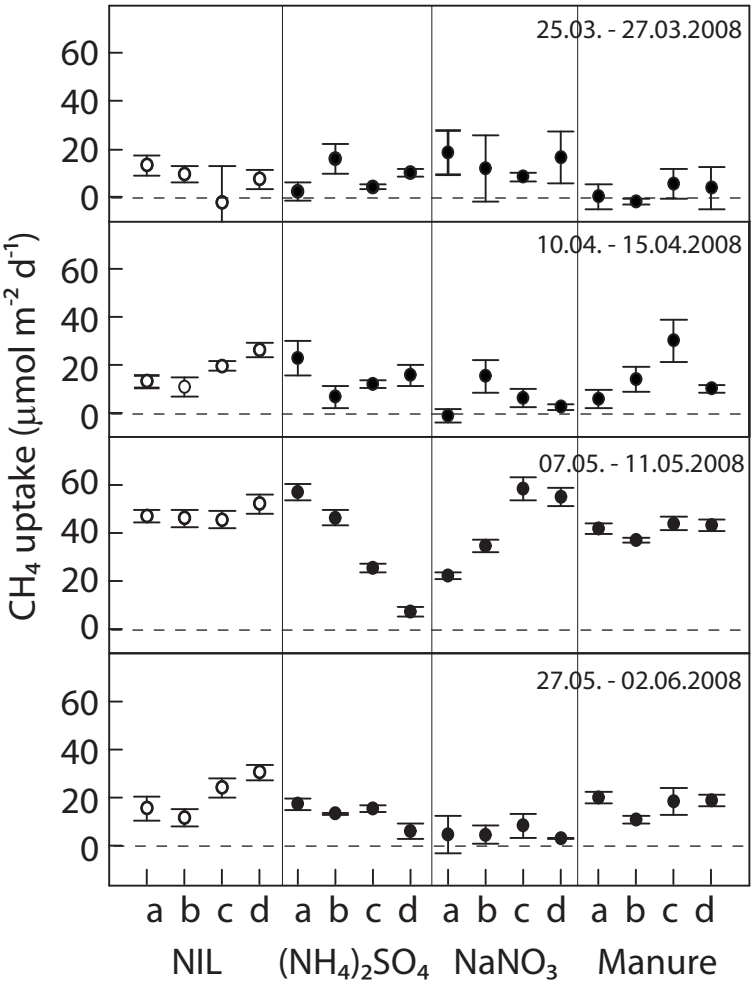

Figure 4.1: Net CH_4 uptake rates in the Park Grass experiment, in dependence of fertilization and liming treatments (a-d, see method section for details). Fluxes were measured in-situ using static chambers. Error bars indicate standard errors of the repeated samplings of each measurement period.

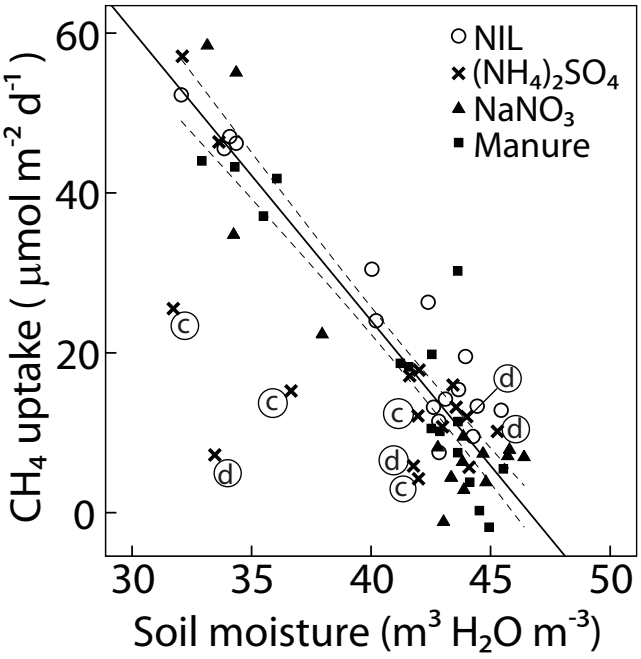

Figure 4.2: Correlation of net CH_4 uptake rates and soil moisture. The plotted data are averages per subplot and measurement period. The solid line shows the correlation between CH_4 flux rates and soil moisture, excluding subplots c and d of the $(NH_4)_2SO_4$-treated soil (R^2=0.9, dashed lines mark the 95 % confidence interval, letters denote subplots).

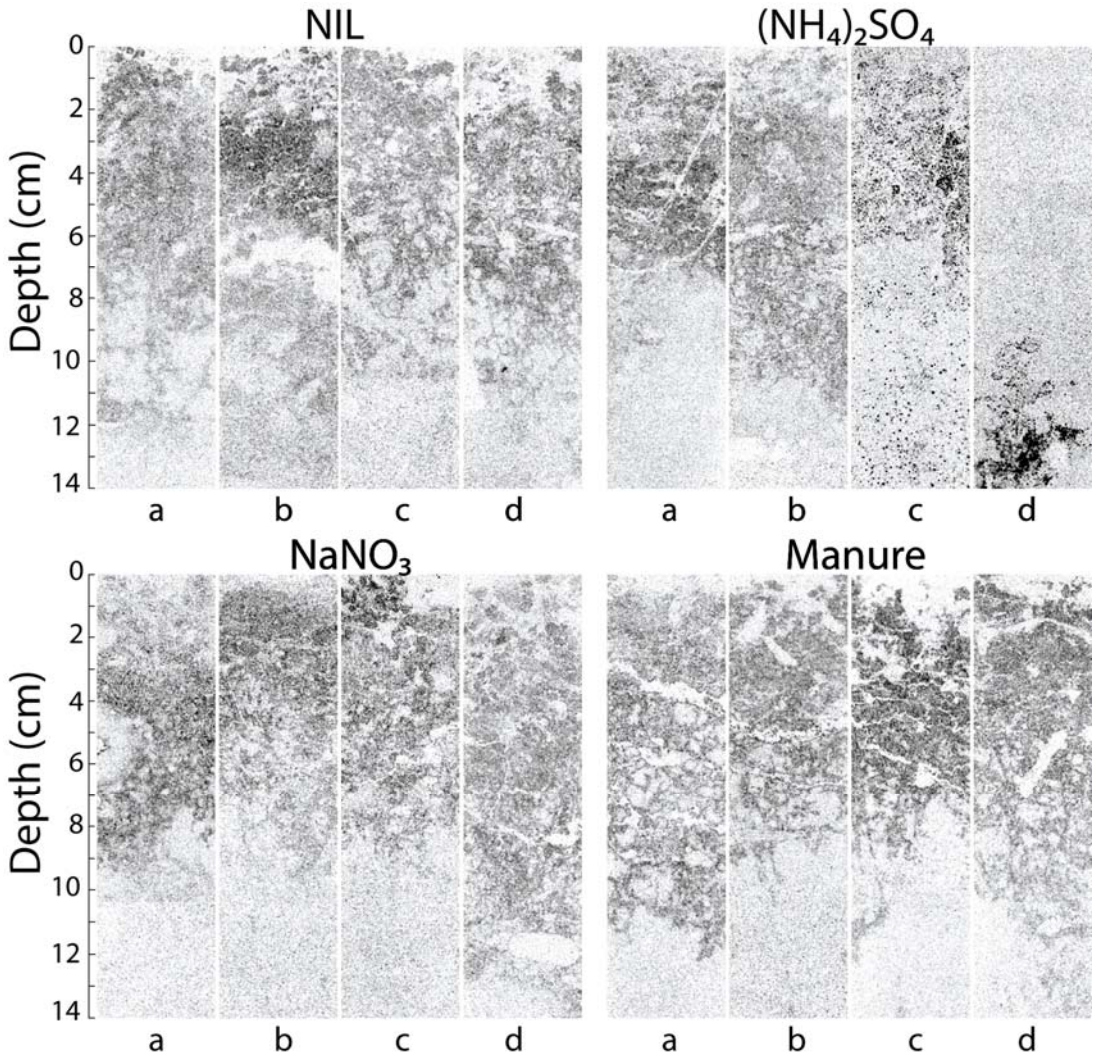

Figure 4.3: Autoradiographic images showing the ^{14}C distribution in soil core sections sampled on the 3rd harvest (11 May 08). The letters a-d indicate the respective subplots (corresponding to liming treatments, see methods section for details). Darker pixels indicate increased ^{14}C assimilation. Note that the data shown is normalized per soil core; for example, the darker pixels at the bottom of the $(NH_4)_2SO_4$-treated core of subplot d do not necessarily correspond to higher methane uptake rates in these spots than in other cores, they only indicate that CH_4 assimilation was restricted to these sites.

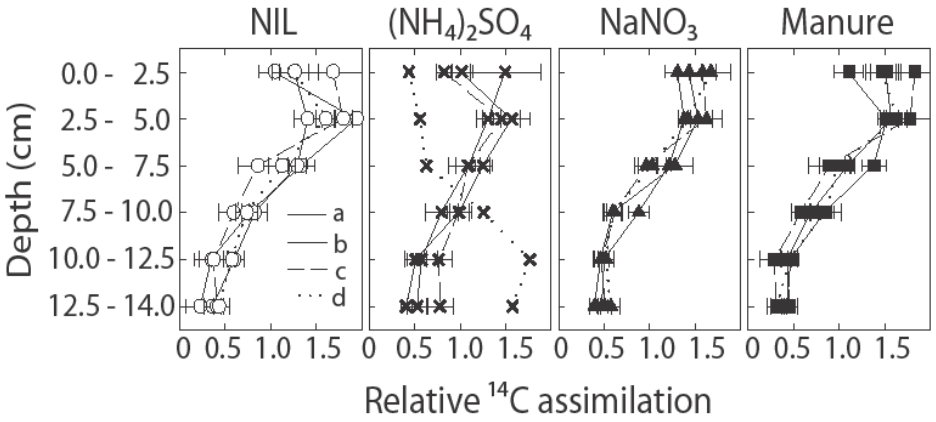

Figure 4.4: Distribution of ^{14}C labeling intensities along the soil profile. Data are normalized per soil core; the figure shows averages and standard errors calculated from the four samplings.

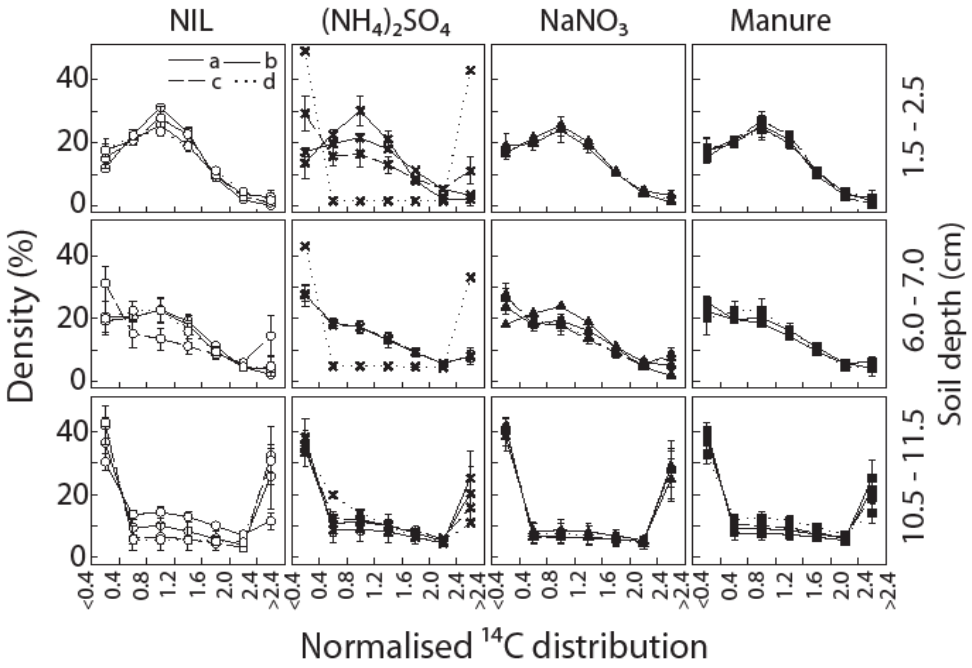

Figure 4.5: Frequency distribution of the label intensity in autoradiographs of $^{14}CH_4$-labelled soil sections of Rothamsted Park Grass plots. All data were normalized per soil core; on a normalized scale, the histogram classes are <0.4, 0.4-0.8, 0.8-1.2, 1.2-1.6, 1.6-2.0, 2.0-2.4 and >2.4. The figure shows means of the four samplings and the corresponding standard errors.

Chapter 5

Synthesis

5.1 Research aims

Methane (CH_4) belongs to the most important greenhouse gases (Introduction, chapter 3). Terrestrial CH_4 sinks contribute up to 10% to the uptake of annually emitted CH_4 (Smith *et al.*, 2000). The process of CH_4 uptake in soils is essentially driven by CH_4 assimilating soil microorganisms (methanotrophic bacteria; Bédard & Knowles, 1989; Conrad, 1996; Hanson & Hanson, 1996). CH_4 assimilation depends mainly on soil aggregation, moisture, pH and ammonium (NH_4^+) concentrations (Dunfield, 2007). However, underlying mechanisms are not completely understood, for example effects of ammonium deposition or pH on CH_4 uptake in soils have been observed only occasionally. Soil physical and chemical properties affecting terrestrial CH_4 uptake vary largely within the soil and little information is available about the spatial distribution of methanotrophic bacteria in intact soils. Thus, the evaluation of soil CH_4 uptake is complex.

The aims of this thesis were

i.) to develop a laboratory protocol, which allows studying the spatial distribution of atmospheric CH_4 uptake in intact soils by visualizing $^{14}CH_4$ assimilation

ii.) to investigate the spatial distribution of CH_4 assimilation in relation to the soil depth, soil aggregates, moisture, humus layers, stones, roots, pH, drought and nitrogen fertilization

iii.) to assess the climatic relevance of distinct spatial distributions of CH_4 uptake by analyzing them in combination with CH_4 net flux data.

5.2 Outcomes

In the course of this thesis a newly developed imaging approach enabled us to two-dimensionally picture CH_4 assimilation in the undisturbed soil at the small scale. Methanotrophs were labelled with $^{14}CH_4$ in intact soil cores which were then freeze-dried, embedded in epoxy resin and cut into soil sections. Autoradiographic images with a resolution of 50 µm were generated from these sections. All known approaches to study CH_4 assimilation in soils (e.g. the use of ^{13}C labelling of phospholipid fatty acids (PLFA), quantitative polymerase chain reaction (PCR), ^{14}C labelling followed by burning of soil particles and subsequent quantification of the label using liquid scintillation counting) so far were not appropriate to link information about either the number or *taxa* of methanotrophic bacteria or CH_4 assimilation activities to the spatial arrangement of particles within the soil. The autoradiographic images of the present work thus provides previously unknown information where in the soil atmospheric CH_4 has been assimilated, while information about the relative amount of the assimilated CH_4 within a single soil core can be derived from the very same images.

Our results reveal that the spatial distribution of CH_4 assimilation directly depends on soil aggregation and the spatial arrangement of those aggregates within the soil, as these impacted CH_4 diffusion and the spatial distribution of liquids such as precipitation and liquid fertilizers in the soil. Nitrogen fertilizers inhibited CH_4 assimilation only in a part of the soil whereas in other parts of the same soil microbes assimilated even increased amounts of CH_4. Results suggest that the extent to which nitrogen fertilizers affect the CH_4 sink not only depends on inhibition of CH_4 uptake by the fertilizer, but also on how much CH_4 is consequently assimilated in other parts of the soil (chapter 3). Effects of fertilizers on the net CH_4 uptake might appear delayed or continuously increase, as the zone with inhibited CH_4 uptake expanded with time. Parts of the soil in which CH_4 uptake was not reduced by the fertilizers were mainly parts of the subsoil. When soil moisture in the subsoil was high, local CH_4 uptake rates were low because CH_4 diffusion is slower in water compared to the soil gas phase. However, despite this negative impact of water on the CH_4 sink, moisture also appeared to reduce the amount of the nitrogen fertilizer that is required to inhibit CH_4 uptake in fertilized soils.

Consistently low amounts of CH_4 were assimilated in the humus layers of soils (chapter 2). If soils were treated with ammonium based fertilizers, humus layers might partly prevent infiltration of ammonium into the uppermost mineral soil, the layer where major CH_4 assimilation occurred. Consequently, CH_4 uptake of such soils might be inhibited by the fertilization treatment to lesser extent as if soils are lacking a humus layer.

NH_4^+ based fertilizers might inhibit CH_4 uptake particularly when soils are acidic or dry. Results further suggest that inhibition of CH_4 uptake depends on the concentration of free NH_4^+ and therefore processes involved in NH_4^+ dynamics also impact CH_4 assimilation (chapters 3 and 4). The NH_4^+ concentration of soils increases when drought reduces NH_4^+ uptake by plants or if nitrification rates are slowed down as a consequence of soil acidification. Results from Hartmann *et al.* (2010) revealed that the CH_4 sink recovers within one to two weeks after fertilization whereas our results suggest CH_4 uptake inhibition in the upper part of the soil to last for longer. Although short-term application of NH_4^+ based fertilizers appear to have little effects on the CH_4 sink, long-term applications might have a major effect on the CH_4 sink, as the part of the soil in which CH_4 is inhibited increases. Soil acidification by NH_4^+ based fertilizers progresses with continuous fertilization (Johnston *et al.*, 1986). In the long-term experiment at *Rothamsted* we were able to show that ammonium sulphate increasingly affected CH_4 uptake of soils with decreasing pH. While in soils with an adjusted pH of 5.0, CH_4 assimilation partly recovered when fertilization was terminated, this appeared not to be the case in soils with pH 4 (chapter 4). We conclude that prolonged application of NH_4^+ based fertilizers will increasingly affect the CH_4 sink, if the underlying inhibitive processes can not be stopped. In summary, we suggest that in order to maintain the soil CH_4 sink, ammonium based fertilizers should not be applied for years to dry soils and liming should be done to acidifying soils.

If applied at high amounts, NH_4^+ releasing fertilizers might significantly reduce CH_4 assimilation in even moist soils. When soil moisture is high, the effect is mainly restricted to inorganic fertilizers. Hütsch *et al.*, (1994) reported that long-term applications of ammonium sulphate reduced CH_4 uptake of soils severely at even lower amounts (96 kg N ha^{-1} yr^{-1}). We conclude that intensive organic nitrogen

fertilization is more effective to reduce global warming than intensive inorganic nitrogen fertilization. Our results further suggest that excrements from cattle severely reduce the CH_4 sink of moist soils.

5.3 Outlook

In chapter 3, we investigated the effect of short-term applied fertilizers on CH_4 uptake on one sample per plot. Results from net flux measurements at the same site revealed that nitrogen fertilizers reduced CH_4 uptake temporarily (Hartmann *et al.*, 2010). In chapter 4 we sampled soil four times during six months and, again, results indicated that the spatial distribution of CH_4 uptake underlay temporal dynamics. Time series analysis of soil samples using the methods applied in this thesis constitutes an appropriate approach to study temporal dynamics of CH_4 uptake in nitrogen fertilized soils. However, samples should be taken more frequently and within shorter periods than we did in the study at the *Rothamsted* Park Grass experiment (chapter 4).

The spatial distribution of CH_4 uptake in the soil in relation to soil features such as roots or stones was further investigated in this thesis comparing autoradiographic images with conventional photographic images of the respective soil cores. More detailed information about how CH_4 assimilation is linked to the soil structure, i.e. the density of the soil particles where CH_4 assimilation occurs, could be gained by combining our method with micro-computed tomography (CT) analysis. We tested micro-CT analysis of unlabelled soil sections embedded in epoxy resin at the *Institute for Snow and Avalanche Research* (SLF) in Davos, Switzerland in collaboration with Dr. Martin Schneebeli and three-dimensional images of soil cores were generated. These images showed soil components within a defined density range with respect to the spatial arrangement of the soil particles. The following approach to study the three-dimensional distribution of CH_4 assimilation with respect to soil structure could be performed: Methanotrophs in intact soils would be labelled with $^{14}CH_4$ and samples could be embedded in epoxy resin as described within this thesis. Subsequently, samples could be analyzed using micro-CT. Finally, after cutting and grinding of the same samples, autoradiographic images could be generated and then be merged with the respective section of the micro-CT image.

Information about methanotrophic biomass in the soil with or without respect to methanotrophic *taxa* can be gained using PLFA or quantitative PCR (Maxfield *et al.*, 2006; Kolb *et al.*, 2005). It would be interesting to combine these methods with our imaging approach by analysing neighbouring soil samples in order to study which *taxa* of methanotrophs or how much metanotrophic biomass is actually involved in the CH_4 assimilation within the soil. However, both methods, PLFA and PCR need further development regarding their applicability on methanotrophs living in upland soils as detailed information about the PLFA as well as genetical information with consideration of the whole diversity of methanotrophs is still lacking.

The approach used in the present work is also convenient for investigations of other ecological processes which can be studied by using β-emitting radioisotopes as tracers. Tracers must though have a half life (hl) of at least two to three weeks as this time is required for the preparation of the soil sections and exposure of the image plates. Convenient tracers are for example ^{35}S (hl = 87 d) and ^{33}P (hl= 25 d). Very recently, our approach was used in a cooperative PhD project at the *Swiss Federal Institute for Forest, Snow and Landscape Research* (WSL) to study the fate of dissolved organic carbon in forest soils.

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Acknowledgements

The time of my PhD was very exciting and I am deeply grateful for the knowledge, the experience or just the time that various persons shared with me.

Thank you, **Pascal Niklaus**, for giving me the opportunity to do this PhD, for guiding me into and through this challenging and diversified work, for giving me technical support and teaching me in writing scientific documents. Thank you for the valuable discussions: even though we did not always agree on each others arguments, they significantly supported this work (with the word "significantly" in a non-statistical context…). I am especially grateful to you for enabling me the work at *Rothamsted, UK*, which was a great and unique experience for me.

Thank you, **Nina Buchmann**, for having me as a PhD student and for advising me on managing my work. It was interesting to see how you handle so many projects by reducing them to the essentials and despite all this work you never forgot people behind the research and to support them.

Thank you, **David Powlson**, for being my co-referent, for the access to the *Rothamsted Experiments* and for reading my texts even though there was not much time. Your passion for science is very catching and I regret that there was no further time for personal scientific discussion.

Thank you, **Ellen Kandeler**, for providing ideas for this thesis and for enabling my visit in your research group where I learned how to do thin sections. I am also grateful for your inputs in the manuscripts.

Thank you, **Patrick Schleppi,** for providing me all your CH_4 flux data from *Alptal*, for taking me to this study site and also for your inputs in the manuscript.

Thank you, **Paul Poulton**, for your scientific information and instructions in context with the *Rothamsted* Park Grass experiment. Thank you for the technical and administrative support at *Rothamsted* and for providing pH and other data from the plots we considered in our research.

Thank you, **Adrian Hartmann**, for all the site management at *Alp Weissenstein* and *Früebüel*, for the sample analysis with gas chromatography, for providing additional data of these two sites and for all the discussions about our research topic but also about the PhD life.

My further acknowledgements go to the following persons:

Martin Schneebeli for the collaborative work with micro-CT analysis. It is a pity that this work could not be integrated in this thesis.

Keith Goulding for giving me access to the *Rothamsted Experiments* and for the hospitality at *Rothamsted Research*.

Patrick Flütsch, Peter Plüss, Annika Ackermann, Carla Soliva, Leo Meile, Annina Zihler, Carmen Kunz, Samuel Bucherer, Martin Schroth, Christine Stein, Sven Marhan, Christian Poll, Daniela Saurer, Patrick Spielmann, Roland Wenger, Paul Borer, Frowin Pirowino, Andreas Jallas and **Jan Giessbrecht** for providing or explaining the handling of equipment and for the help with test or final experiments.

Sarah Wernicke for helping me grinding the soil sections. I guess this work was the dirtiest of all - this needs to be mentioned in a separate line.

Lutz Merbold and **Ansgar Kahmen** for revising my texts and for the gentle comments.

Mario, Rebecca, Lisa, Ivan, Mariana, Imma, Nicholas and Alistair for their support at *Rothamsted*: for helping me with logistics, introducing me to the vegetation and the insects in the Park Grass experiment and the surrounding and mostly for giving me a second home in the manor house.

To the current and former members of the Grassland-group at ETH, especially to those who were always there for me. During the time of my PhD, I observed how people in this group more and more collaborated and supported each other. This is so much motivating!

I especially want to thank **Daniel Stiehl**. Daniel, you supported me in so many ways. Thank you for inspiring me, for giving me practical advice and technical and writing support. You reduced my doubts and fears. The numerous philosophical discussions with you about science and research, what it is, what it should be like and how it could be improved, they were so valuable to me and supported me in doing my work.

Family and **friends**, thank you all for your personal support and being with me.

This work was founded by Swiss National Science Foundation grant 315230-112681 and during the last four months by the Grassland Science Group, Nina Buchmann. The Walter Hochstrasser Stiftung is acknowledged for funding of my travels to the *Rothamsted Research Experimental Station*.